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Regulation Of Proline-Directed Phosphorylation Site, Threonine 53 On Dopamine Transporter

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REGULATION OF PROLINE-DIRECTED PHOSPHORYLATION SITE, THREONINE 53 ON DOPAMINE TRANSPORTER

By

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This dissertation, submitted by Sathyavathi Challasivakanaka in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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This dissertation is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

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Dean of the School of Graduate Studies

October 30, 2014
Date

PERMISSION

Title	Regulation of proline-directed phosphorylation site, threonine 53 on dopamine transporter
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ABBREVIATIONS

AD	Alzheimer's disease
ADHD	attention deficit hyperactivity disorder
AMPH	amphetamine
BZT	benztropine
CaMKII	Ca^{2+}/calmodulin-dependent kinase II
CNS	central nervous system
COC	cocaine
COMT	catechol-O-methyltransferase
D_2Rs	dopamine D2 receptors
DA	dopamine
DAT	dopamine transporter
dDAT	drosophila DAT
DOPA	dihydroxyphenylalanine
ERK	extracellular signal-regulated kinase
FKBP	FK506 binding proteins
hDAT	human DAT
HVA	homovanilic acid
JNK	c-Jun N-terminal kinase
KO	knock out
KRH	Krebs-Ringer/HEPES
Leu T	leucine transporter from Aquifex aeolicus
LLC-PK_1	Lewis lung carcinoma porcine kidney cells
MAO	monoamine oxidase
MAPK	mitogen activated protein kinase
METH	methamphetamine
NAC	nucleus accumbens
N-DAT	DAT N-terminal tail fusion protein
NET	norepinephrine transporter
NIMA	never in mitosis A
OA	okadaic acid
o.w.w	original wet weight
P	phospho
PI-3K	phosphatidylinositol 3-kinase
PICK-1	PDZ domain-containing protein interacting with C kinase
Pin1	protein interacting with NIMA
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PP	protein phosphatase

PPIases	prolyl isomerases
PVDF	polyvinylidene diflouride
rDAT	rat DAT
RIPA	radioimmunoprecipitation assay
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERT	sertonin transporter
SLC6	solute carrier 6
Syn1A	Syntaxin1A
TMs	transmembrane-spanning domains
T	threonine
VMAT	vesicular monoamine transporter
VTA	ventral tagmental area

ACKNOWLEDGEMENTS

I would like to begin by thanking my advisor Dr. Roxanne Vaughan for her immense support through out my graduate program at UND. Her encouragement and continuous guidance has played a prominent role in my development as a scientist. Dr. Vaughan has taught and challenged me to critically think and plan experiments with efficient controls. Her positive outlook and support has made me overcome scientific challenges. Her enthusiasm for science is contagious. She is one of the few people I know who can balance professional and personal life perfectly. Dr. Vaughan is a perfect scientific role model and I am grateful to be trained by her. She encouraged me to attend several national and state level conferences, which helped me develop the ability to present research to a wider audience. I appreciate the time she dedicated for mentoring in scientific-writing and oral presentation skills. I am forever grateful for the scientific training, encouragement and support that Dr. Vaughan has provided me for the past 5 years.

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To amma and nandi.

ABSTRACT

The dopamine transporter (DAT) is a plasma membrane protein that clears extraneuronal dopamine (DA) and thus controls the spatio-temporal dynamics of dopaminergic neurotransmission. Also, DAT is the major target for psychostimulant substrates, amphetamine (AMPH) and methamphetamine (METH), and psychostimulant uptake blocker, cocaine (COC). DAT is a phosphoprotein with both the N- and C-termini facing toward the cytosol, with multiple phosphorylation sites on the N-terminus. DAT has a closely spaced 6-serine cluster on the distal N-terminus that is phosphorylated in a protein kinase C (PKC)-dependent manner and a recently identified proline-directed site, Thr (T) 53, that is phosphorylated *in vitro* by the MAP kinases ERK, JNK and p38. Current studies indicate that COC and AMPH impact DAT regulatory properties including uptake activity and surface expression. Although the mechanism of drug action on DAT is not completely known, phosphorylation conditions of DAT have been found to be associated with altered surface expression and activity of DAT. In this study, we examined the effect of several psychostimulant drugs on the phosphorylation of DAT using a newly developed phospho-specific antibody against phosphorylated T53 (pT53) on DAT. A detailed analysis of pT53 on DAT was performed in LLC-PK_1 cells expressing rat DAT (rDAT), rat striatal synaptosomes and *in vivo* in male Sprague-Dawley rats. Our studies revealed psychostimulant substrates but not uptake blockers significantly stimulated pT53. Pretreatment with COC blocked the AMPH stimulation of pT53 indicating that AMPH stimulates pT53 in a DAT-dependent manner. In rat striatal synaptosomes, METH-stimulation of pT53 was very fast; occurring within 60 sec.

Subcutaneous injections of METH in rats stimulated pT53 in a time-dependent manner. Our study demonstrates that the proline-directed phosphorylation site, pT53 is subject to differential regulation by psychostimulant drugs. Prolyl cis-trans isomerase, Pin1 catalyzes the cis-trans isomerization of pThr-Pro peptides allowing the dephosphorylation by conformation-specific phosphatases. We investigated the regulation of pT53 by Pin1 using Juglone (Jug), a small molecule inhibitor of Pin1. Treatment with Jug in LLC-PK_1 rDAT cells and rat striatal synaptosomes revealed significant stimulation of pT53. Jug treatment enhances [^{3}H]DA efflux from rat striatal synaptosomes. ELISA indicated interaction between the N-terminus of DAT and Pin1. This is the first evidence of DAT regulation by Pin1. Our study demonstrates the regulation of DAT pT53 by Pin1 and psychostimulant drugs under physiological conditions.

CHAPTER I

INTRODUCTION

Neurotransmission

The brain governs physiological and psychological processes of the human body through a highly complex and coordinated network composed of billions of neurons. Each neuron communicates with other neurons by a process known as neurotransmission. Neurotransmission occurs at specialized neuronal junctions called synapses. This process involves the release of neurotransmitter from the presynaptic neuron, which is recognized by the receptors on the surface of the postsynaptic neuron.

Synaptic vesicles that serve as the storage pool of the neurotransmitters are docked at the active zones of presynaptic neuronal membranes and primed for the release of neurotransmitter. Neurotransmission occurs in response to the depolarization of the presynaptic neuronal membrane upon arrival of the action potential. The action potential that originates in the cell body of the neuron travels down the axon terminal and opens Ca^{2+} channels. The increase in Ca^{2+} triggers the fusion of the primed synaptic vesicles with the neuronal membrane to release their contents into the synaptic cleft. The released neurotransmitter is sensed by the receptors on the postsynaptic neuron propagating the signal to the connecting neuron. Neurotransmission is terminated by clearance of the neurotransmitter from the synapse in one or more of the following ways- enzymatic

degradation, diffusion and or re-uptake of the released neurotransmitter in to presynaptic neuron by neurotransmitter-specific transporter proteins or re-uptake into a neighboring glial cell [1].

Dopamine and dopaminergic system

Dopamine (DA) is synthesized in dopaminergic neurons from tyrosine. Tyrosine is also the precursor of other catecholamine neurotransmitters like norepinephrine (NE) and epinephrine. Initially DA was only considered as a mere precursor of NE and epinephrine. Later, neurotransmitter properties of DA were demonstrated and since then DA is regarded as a neurotransmitter which controls movement, reward-seeking behavior, emotion and cognition [2,3]. Dopaminergic neurons are present in the ventral tegmental area (VTA), the substantia nigra and the arcuate nucleus of the hypothalamus regions of the brain. There are four dopaminergic pathways, mesocortical, mesolimbic, nigrostriatal, and tuberoinfundibular (Fig.1). Among the four pathways, the mesolimbic pathway that connects the VTA of the mid brain to the nucleus accumbens in the striatum is associated with a reward circuit and known to be involved in the drug addiction process [4].

Dopamine transporter

Dopaminergic neurotransmission is terminated by various processes- uptake of DA by glial cells and dopaminergic neurons, degradation of DA by catechol-O-methyl transferase (COMT) and monoamine oxidase (MAO). The major process of dopaminergic signaling termination occurs by transporter proteins localized to the presynaptic neuronal membrane. DA is rapidly transported into the presynaptic neuron by

Figure 1. The four dopaminergic pathways

Dopaminergic neurons extend from the ventral tegmental area (VTA), substantia nigra and mesobasal hypothalamus to the nucleus accumbens, prefrontal cortex, striatum and base of the hypothalamus. These neuronal projections form the mesolimbic, mesocortical, nigrostriatal, and tuberoinfundibular pathways.

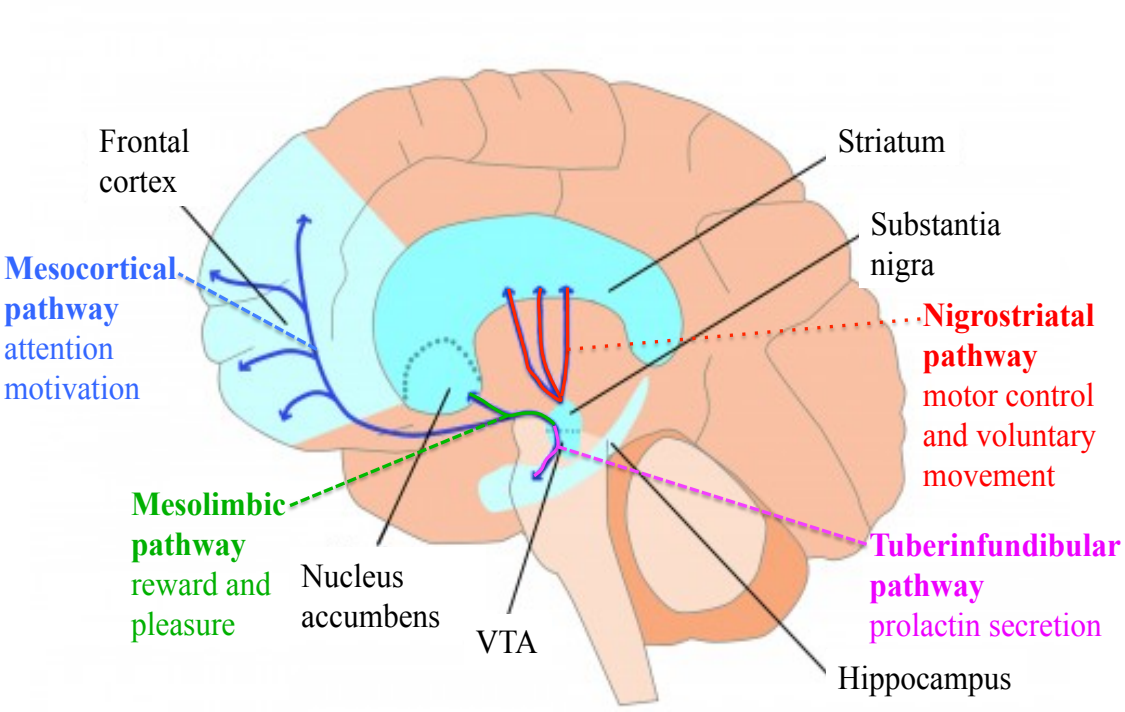

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Figure 1. The four dopaminergic pathways

transporters localized to the presynaptic neuronal membrane. The transporters are neurotransmitter-specific dopamine transporter (DAT) for DA, serotonin transporter (SERT) for serotonin, norepinephrine transporter (NET) for norepinephrine [5].

Primary target for psychostimulants

Psychostimulants like methamphetamine (METH), d-amphetamine (AMPH) (collectively referred to as amphetamines) and cocaine (COC) (Fig.3), have been a major threat to society. The strong reinforcing properties and the abuse potential of these drugs indicate the requirement for development of an effective medication to treat drug addiction. These psychostimulant drugs affect the neural circuitry and increase cravings for repeated drug use leading to addiction [6]. Understanding the molecular mechanisms of psychostimulant drugs for the development of therapeutic approach has been an active area of research.

The psychostimulants are known to elicit their actions primarily by altering dopaminergic homeostasis in the brain (Fig.2). In 1980s, a few studies implicated that dopamine-containing neurons in the forebrain projections and the ventral tagmentum of the brain region are crucial for the reinforcing properties of psychostimulant drugs like COC and AMPH [7]. Though the role of dopaminergic signaling in psychostimulant abuse was not completely established, pathology of the dopamine system was predicted as a potential target for psychostimulant action [4], [7]. The requirement of DAT and DA signaling in psychostimulant action was strongly established with the development of

Figure 2. Representation of a dopaminergic synapse under physiological and pathological states

A. Under physiological conditions, DA is recycled back into the presynaptic neuron by DAT where DA is transported into vesicles by VMAT and stored. B. In the presence of the DAT blocker, cocaine, the reuptake of DA into presynaptic neuron is prevented resulting in buildup of DA at the synapse. C. Amphetamines are DAT substrates, once inside the neuron, they also get transported via VMAT into vesicles and cause the release of DA increasing the cytosolic concentration of DA and cause non-vesicular release of DA via DAT.

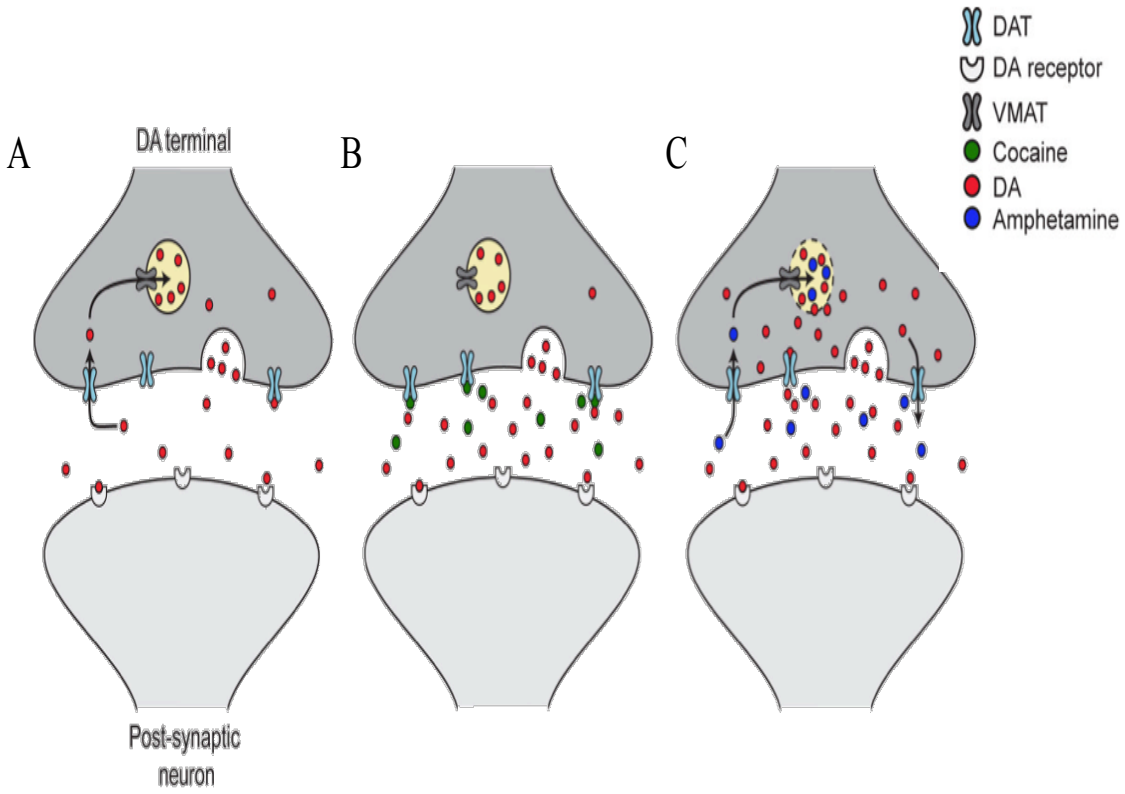

Image courtesy, R. A. Espana and S. R. Jones, 2013 Presynaptic dopamine modulation by stimulant self-administration. *Front. Biosci. (Schol. Ed).*, vol. 5, pp. 261–76, with permission [8]

Figure 2. Representation of a dopaminergic synapse under physiological and pathological states

Figure 3. Chemical structures of dopamine transporter substrates and cocaine

Representation of chemical structures of dopamine transporter (DAT) substrates: dopamine, amphetamine, methamphetamine, MPP^+ and DAT blocker: cocaine

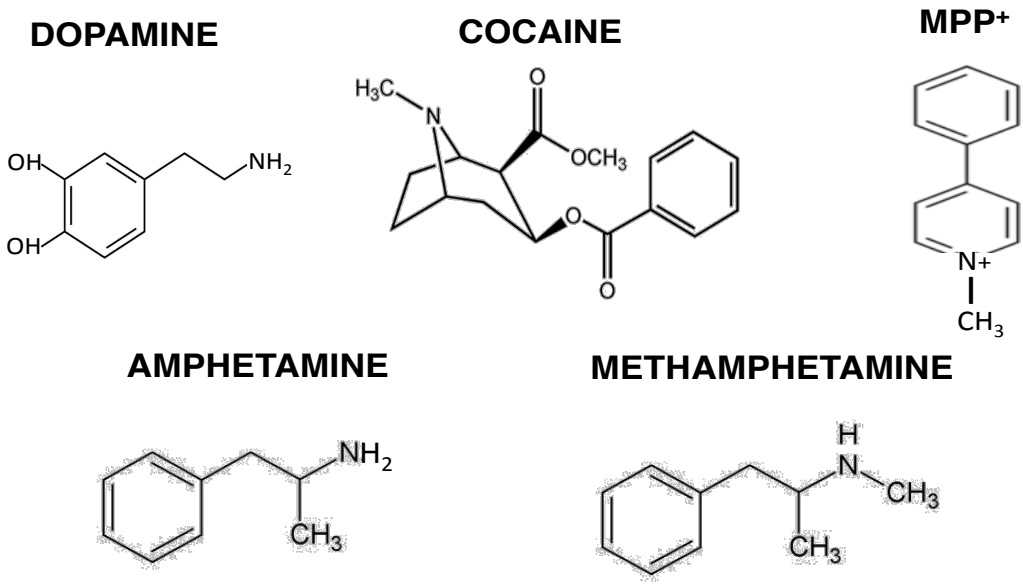

Figure 3. Chemical structures of dopamine transporter substrates and cocaine

DAT knock out (KO) mice. Homozygous DAT KO mice displayed decreased DA uptake and highly compromised ability to clear the extraneuronal DA consistent with increased spontaneous locomotor activity [9]. This further led to the 'dopamine hypothesis', according to which, cocaine first binds to DAT and blocks DA uptake (Fig.2), which results in potentiation of dopaminergic neurotransmission in the limbic pathways. This ultimately leads to reinforcement of the behavior that is associated with the molecular events of cocaine binding and uptake inhibition [10].

COC is non-transportable through DAT [11], the psychostimulants and other therapeutic drugs that belong to this class are called blockers. DAT blockers that do not induce cocaine-like behavioral patterns such as benztropine (BZT) are termed atypical uptake blockers [12]. Along with the inhibition in DA uptake, DA receptors are also implicated in the reinforcement of COC [7].

Amphetamines are also a prescribed medication for ADHD and depression. Amphetamines act in a complex fashion to impact DA signaling. In addition to uptake inhibition of DAT [13], AMPH also gets transported via DAT into the presynaptic neuron. Once inside the presynaptic neuron, AMPH causes reverse transport of DA via DAT (Fig.2). This process is also referred to as efflux. In addition, at higher concentrations, amphetamines are also known to diffuse through the membrane due to their hydrophobic nature. Pretreatment with COC blocks AMPH-stimulated DA efflux indicating that DAT-mediated entry of AMPH is required for the efflux mechanism [14, 15]. The mechanism of DAT transport reversal by AMPH is proposed to occur by an exchange diffusion model. According to this model, AMPH gets transported into the presynaptic neuron in a Na^+-dependent manner to cause reverse transport of DA through

DAT. AMPH also contributes to the increased synaptic DA concentration by depleting the secretory vesicle stores of DA. In addition, AMPH also inhibits MAO preventing the oxidation and inactivation of DA [16]. These mechanisms result in the increase in the cytosolic concentration of DA favoring the AMPH-stimulated efflux and contributing to oxidative damage. In addition to psychostimulant drugs, DAT also serves as a gateway for several environmental toxins like MPP^+, which is derived from MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) by MAO [17][18] (Fig.3).

Structure

DAT, NET, and SERT along with transporters for other solutes like glycine and GABA are encoded by genes of the SLC6 (Solute Carrier 6) family. The members of the SLC6 family are expressed in a wide range of tissues with major distribution in the central nervous system (CNS) where they function to maintain homeostasis of neurotransmitters. These are secondary active transporters that utilize the electro-chemical gradient generated by $Na^{+/}K^+$ ATPase to drive the symport of neurotransmitter and Na^+ [19] [5] [20] [21].

The cDNA of rat DAT was first cloned, transiently expressed and characterized for DA uptake activity in HeLa cells [22]. Human DAT (hDAT) is a 620 amino acid protein while rat DAT (rDAT) is comprised of 619 amino acids, and both contain 12 transmembrane (TM) domains with both N and C termini facing towards the cytosol. The primary sequence of DAT has revealed sites for glycosylation on the extracellular loop which were later observed to have a role in regulating function, surface expression [20] [23]. Though the hydrophobicity analysis and mutation studies revealed 12 TMs and the

glycosylation sites, the three dimensional structure of DAT was not known until the crystallization of a bacterial homologue, a leucine transporter (Leu T) from *Auifex aeolicus* [24]. This bacterial homologue shares 20% sequence homology with DAT. Leu T crystal structure displays a pseudo-symmetrical arrangement of TMs 1-5 and TMs 6-10 with TMs 1, 3, 6 and 8 constituting the core of the transporter forming the substrate translocation pathway. In addition to the information from the Leu T crystal structure, drosophila DAT (dDAT) crystal structure was recently reported [25].

DAT is proposed to function by an alternating access mechanism [26] in which DAT undergoes a series of conformational changes from an outward-facing conformational state to an inward-facing conformation (Fig.4). The different conformations of DAT are stabilized by different gates, which allow access to DAT from either the extracellular or intracellular side [27]. The outward facing conformation of DAT in which the extracellular gate is open, allows the substrate to bind from the extracellular side. The extracellular gate is formed by a salt bridge between the amino acids R85 on TM1 and D476 on TM10. The intracellular gate formed by R60 on the N-terminus and D436 on TM8 opens to form the inward-facing state [24][28]. This conformation allows the release of substrate to the intracellular milieu. The substrate-bound transporter with both the extracellular and the intracellular gates closed results in an occluded conformation.

Figure 4. Alternating access model of SLC6 transporters

The figure depicts the series of conformational states in an alternating access model of SLC6 transporters. The outward-facing conformation allows substrate and sodium ion binding to the transporter. The occluded conformation is then followed representing a state in which, the extracellular and the intracellular gates are closed. The inward facing conformation with the intracellular gate open allows the release of the substrate and the co-transported ions into the cytosol.

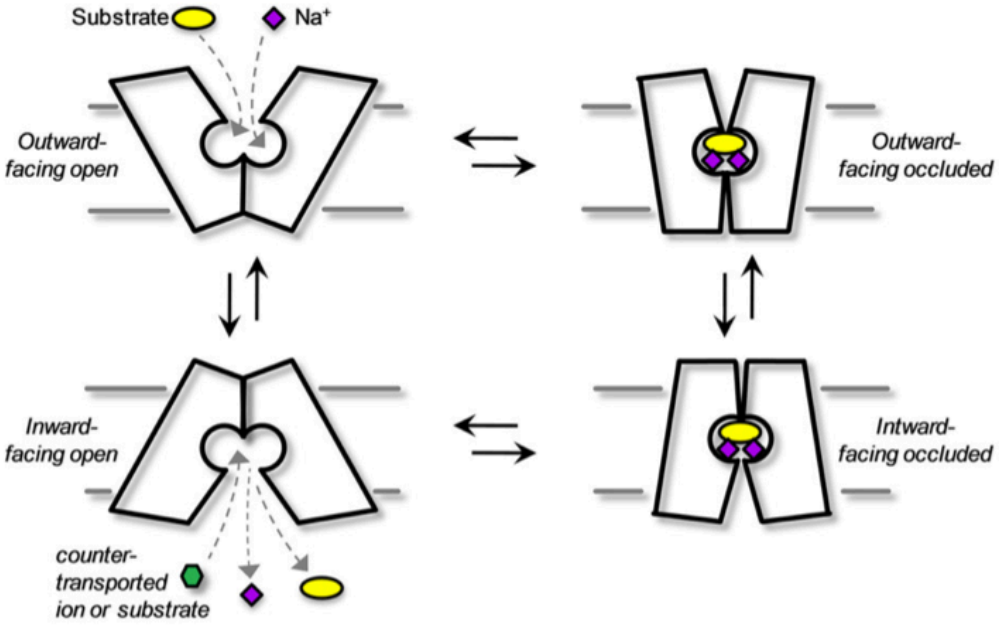

Image courtesy, A. S. Kristensen, J. Andersen, T. N. Jørgensen, L. Sørensen, J. Eriksen, C. J. Loland, K. Strømgaard, and U. Gether, 2011 SLC6 neurotransmitter transporters structure, function, and regulation. *Pharmacol. Rev.*, vol. 63, pp. 585–640, with permission [27].

Figure 4. Alternating access model of SLC6 transporters

All the gating residues are conserved in the SLC6 transporters [24]. Mutation of the gating residues in DAT has been shown to impact the conformational states of the transporter [28] [29]. The intracellular gating resides R60 and D436 in DAT, when mutated to R60A and D436A displayed significant decreases in V_{max} for [^{3}H]DA uptake [28]. The surface expression of both the mutants were comparable to WT DAT suggesting the decreased uptake was not due to altered surface expression but due to the impairment of the intracellular gating residues [28]. The Leu T crystal structure revealed the interaction of the intracellular gating residue, R60 with Y335 in TM6. Y335A DAT displayed reduced V_{max} with no loss in surface expression compared to WT DAT indicating its prominent role in stabilizing the intracellular gate and hence DAT function [29].

The role and requirement of the following residues R60, D436 and Y335 in the intracellular gating mechanism has been further substantiated by Zn^{2+} coordination experiments. Zn^{2+} interacts with H193, H375 and E396 to stabilize the outward facing conformation of DAT [30]. The decreased uptake activities of R60A, D436A and Y335A DATs were rescued in the presence of Zn^{2+}[28], [29]. This demonstrates the ability of Zn^{2+} to stabilize the outward facing conformation and facilitate the uptake activity in intracellular gate impaired DAT mutants.

In addition to the role of Zn^{2+} in stabilizing the outward open conformation of DAT, the dDAT crystal structure revealed the presence of cholesterol, which is hypothesized to stabilize DAT in an outward-open conformation [25]. This indicates that several factors play a role in stabilizing different conformational states of DAT. Also

reported in dDAT structure was a kink in TM12 that resembles a latch facing away from the transporter which is predicted to potentially regulate DAT function [25].

The cytoplasmic tails, both N- and C-termini, are much shorter in both dDAT and the bacterial homologue Leu T leading to the lack of a resolved structure for these tails [24], [25]. Both tails are sites for several post-translational modifications and regulate various characteristics of the transporter while serving as sites of interaction for many binding partners.

DAT interaction partners

DAT has been shown to interact and form complexes with various proteins, which in turn affect the function, phosphorylation, trafficking and, subcellular localization of DAT (Fig.5). DAT is a dynamic membrane protein that trafficks to and from the plasma membrane. Some of the proteins that interact with DAT include synaptic vesicular plasma membrane proteins and enzymes like PP2A, PKC β, RACK, Hic-5, synaptogyrin-3, VMAT2, Syntaxin 1A (Syn 1A) [31]–[34].

Syn 1A, a SNARE protein was observed to decrease DAT uptake activity along with decreased surface expression in a heterologous system co-expressing DAT and Syn 1A [35]. In addition, Syn 1A was also found to enhance AMPH-stimulated DA efflux [36]. This indicates the role of Syn 1A in regulating DAT function under physiological conditions and drug abused states. Membrane micro domain marker, Flotillin 1 (Flot 1) was recently identified as a protein required for PKC-triggered endocytosis of DAT that also favors AMPH-induced efflux in DA primary neurons without affecting DA uptake [37]. Yeast two-hybrid analysis and GST pull-down assays confirmed Rin (Ras like in

Figure 5. Representation of interaction partners of the dopamine transporter

Figure represents the dopamine transporter embedded in a lipid bilayer with the post-translational modifications on both N-terminal and C-terminal tails. The interaction partners of DAT – Rin (blue), CaMK (green), Flotilin1 (dark green) are depicted.

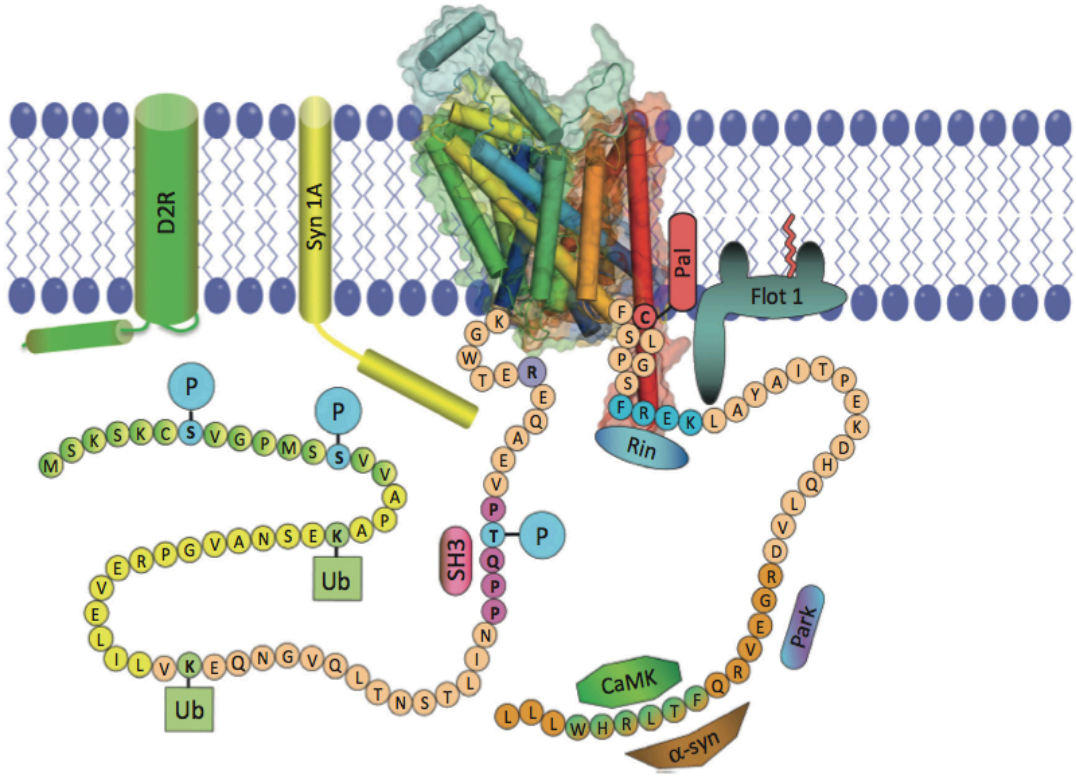

Image courtesy, R. A. Vaughan and J. D. Foster, 2013 Mechanisms of dopamine transporter regulation in normal and disease states. *Trends Pharmacol. Sci.*, vol. 34, no. 9, pp. 489–96, [38].

Figure 5. Representation of interaction partners of the dopamine transporter

neurons) as a DAT interacting protein in a PKC regulated manner and required for PKC-triggered endocytosis of DAT [39]. AMPH is also known to impact the surface expression of DAT.

AMPH-induced internalization of DAT is observed in both heterologous expression system and rat striatal synaptosomal preparations [40]. Interestingly, in the early treatment times (60 sec) [41], amphetamines cause rapid increase in the surface expression of DAT in a heterologous expression system. Methamphetamine (METH) exposure redistributes dopamine from vesicles to the cytoplasm in dopamine neuronal cultures contributing to the formation of reactive species [42] and also triggers DAT complex formation [43]. Involvement of many players in DAT interaction and regulation appears that the system may have evolved with several backup or alternate mechanisms for monitoring DAT activity and surface expression under normal as well as under diseased or drug abused states accordingly.

Regulation of DAT

Phosphorylation

DAT has been shown to be regulated by various protein kinases including PKC, ERK, PKA, and CaMKII [44]–[47] with PKC regulation of DAT being the most extensively studied. In rat striatal tissue and cultured cells stably expressing hDAT or rDAT, both PKC activation with phorbol 12-myristate 13-acetate (PMA) [45] and inhibition of phosphatases with okadaic acid (OA) increases phosphorylation of DAT in a dose-dependent manner [45], [48]. PKC stimulation in native tissue as well as in heterologous

Figure 6. Schematic representation of the rat dopamine transporter

The figure shows the rat dopamine transporter with 12 transmembrane domains connected by extracellular and the intracellular loops with both N- and C-termini facing towards the cytoplasm. Known *in vivo* phosphorylation sites are represented in big light-blue circles. Also depicted, in small light-blue circles, are predicted phosphorylation sites. Depicted in green is epitope 16 on the N-terminus and the palmitoylation site, in brown, on the C-terminus.

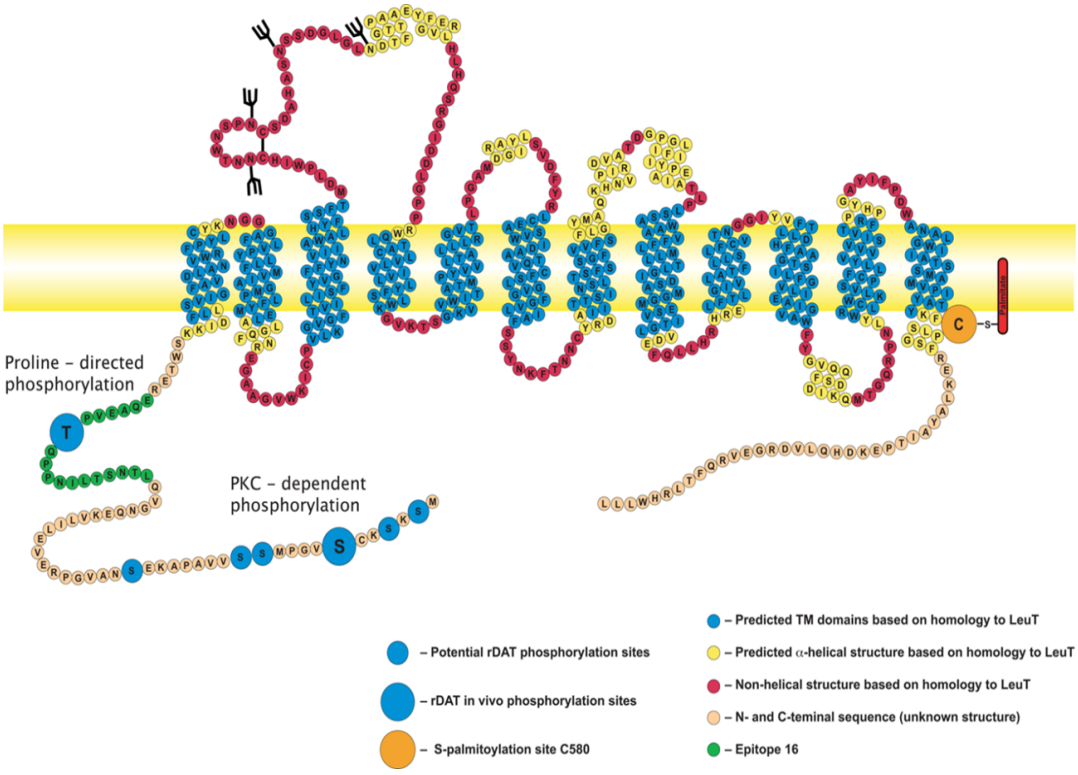

Figure 6. Schematic representation of the rat dopamine transporter

expression systems have shown decreased V_{max} with no change in affinity [45], [49]–[51]. Down regulation of DAT activity in the presence of PKC activation is associated with decreased surface expression of DAT. PKC activation was demonstrated to localize DAT to recycling endosomes where it colocalizes with transferrin receptor [52]. A C-terminal subdomain FREKLAYAIA, spanning amino acids 587-596 of DAT (Fig.6) was later implicated to be required for constitutive endocytosis and PKC-stimulated down regulation of DAT in PC12 cells and primary midbrain neurons. The sequence FREKLAYAIA is conserved among the other neurotransmitter transporters and was demonstrated to be required for a fellow SLC6 transporter, NET [53]. Further studies on this subdomain led to a proposed model that the residues in the first half of the FREKLAYAIA spanning residues 587-590 might act as a braking mechanism to control basal endocytosis, with PKC activation releasing the break resulting in enhanced endocytosis [54]. Although PCK-stimulated down regulation of DAT activity was thought to be mediated through phosphorylation of DAT, truncation of distal N-terminal serines displayed no effect on PKC-stimulated internalization, ruling out the notion of PKC phosphorylation of DAT as an endocytosis requirement and implicating the potential involvement of another protein [55].

Mitogen activated protein kinases (MAPK) have been shown to regulate DAT function and surface expression. Inhibition of MAPK with U0126 or PD98059 significantly decreased DAT function in both rat striatal synaptosomes and in HEK 293 cells stably expressing DAT. The decrease in uptake activity in MAPK-inhibited conditions was associated with enhanced DAT internalization [56]. Later in 2007 another study revealed that DA receptor agonist, quinpirole, increased DAT uptake activity in

N2A and EM4 cells (HEK 293 cells stably expressing macrophage scavenging receptor). The quinpirole treatment also stimulated the activated forms of MAPK and phosphoinositide-3 kinase (PI-3 kinase). Inhibition of both these kinases abolished the agonist-stimulated increase in DAT uptake activity and surface expression. Acute treatment with DA receptor agonists increased DAT surface expression while longer treatment times increased internalization [57].

Metabolic [^{32}P] labeling and phosphoamino acid analysis in rat striatal slices and DAT expressing cell systems have narrowed down the PKC phosphorylation sites to the N-terminal 6-serine cluster with a majority of the phosphorylation and a threonine with a faint phosphorylation signal at the distal N-terminus of DAT [58] (Fig.6) . Though PKC was the extensively studied kinase for DAT phosphorylation, i*n vitro* kinase assays with recombinantly expressed N-terminus of DAT (N-DAT) showed increased phosphorylation with proline-directed kinases: ERK, PKC, P38, JNK. Gorentla et al identified the threonine as a proline-directed phosphorylation site on the DAT N-terminus, T53 [44]. This proline-directed site is also found conserved in human DAT as a serine. The evidence that has accumulated over years that has shown DAT to be phosphorylated by different kinases triggered research in trying to understand the impact of the phosphorylation on function, surface expression and other properties of DAT.

DAT phosphorylation is also affected by psychostimulants like AMPH and METH but not COC. *In vitro* and *in vivo* treatments of amphetamines stimulate phosphorylation of DAT, which is abolished by PKC inhibitors. This phosphorylation was found to be at the serine cluster at the distal N-terminus of DAT as Δ21 DAT, a truncation mutant which lacks the first 21 residues had no basal or AMPH-stimulated

phosphorylation [59]. Some studies have shown that the N-terminus [60] and phosphorylation on the N-terminus of DAT are required for AMPH-stimulated efflux [61]. CaMKII interaction with the C-terminus of DAT has been demonstrated in both heterologous expression systems and in dopaminergic neurons. Interaction of CaMKII with DAT C-terminus facilitates phosphorylation of the N-terminus of DAT which is required for AMPH-stimulated DA efflux. Inhibition of CaMKII or mutation of N-terminal phosphorylation sites on DAT attenuated both AMPH-stimulated DA efflux and MPP^+ efflux suggesting the importance of N-terminal phosphorylation for the efflux mechanism of AMPH [62,63]. PKC activation by PMA increases DAT-mediated efflux indicating either a direct role for DAT phosphorylation in efflux or attraction of an interacting partner that facilitates efflux [64]. Though AMPH induces efflux and stimulates DAT phosphorylation, METH has been observed to have a stronger effect than AMPH [65]. All these studies imply that AMPH-stimulated DAT phosphorylation could be a key mechanism responsible for the psychostimulant action and of the strong reinforcement characteristic of this drug.

Proline-directed phosphorylation

Phosphorylation of a serine/threonine preceding a proline (proline-directed phosphorylation site) renders an additional structural change to the protein [66] and is predicted to impact the structure and function of the protein (Fig.7). Prolyl isomerases (PPIases) catalyze the conversion of cis/trans conformation of Ser/Thr-Pro peptides. Pin1 (Protein interacting with NIMA), a parvulin family member, specifically catalyzes the isomerization of pSer/Thr-Pro motifs [67], [68]. The cis to trans isomerization rate of

Figure 7. Isomerization of proline-directed phosphorylation sites by Pin1

Peptidyl prolyl isomerase, Pin1, catalyzes the cis-trans isomerization of phosphorylated-Thr-Pro (pT-P) allowing the dephosphorylation by conformation-specific phosphatases. Pin1 stabilizes the cis and trans conformations of pT-P of the protein, which are predicted to display different functional characteristics.

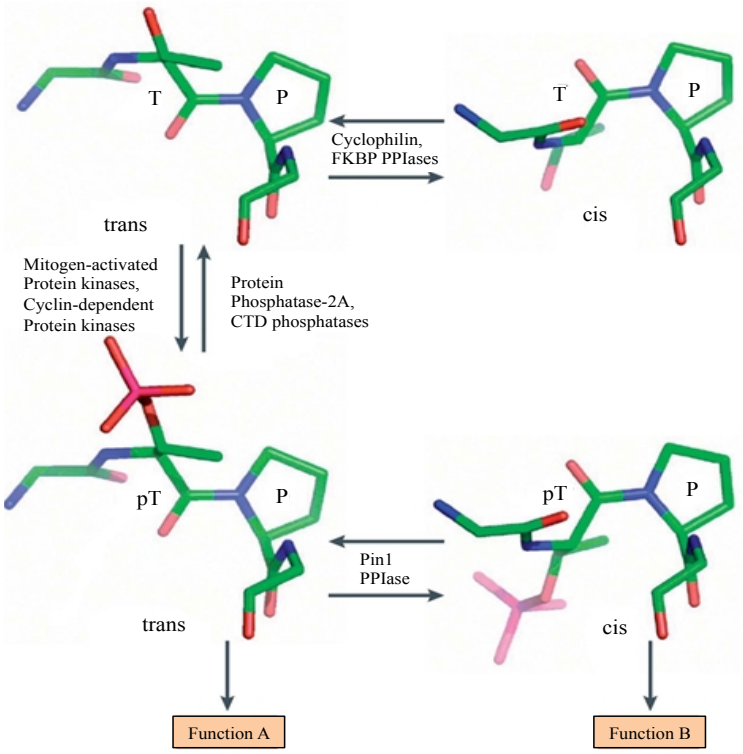

Image adapted from K. P. Lu and X. Z. Zhou, 2007, The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease., *Nat. Rev. Mol. Cell Biol.*, vol. 8, no. 11, pp. 904–16, with permission [68].

Figure 7. Isomerization of proline-directed phosphorylation sites by Pin1

pSer/Thr-Pro-containing peptides is intrinsically slow and requires the activity of the isomerases.

T53 is the only reported proline-directed phosphorylation site on the DAT N-terminus that is phosphorylated *in vitro* by proline-directed kinases such as ERK, JNK and P38 [44]. The importance of T53 in regulating DAT function was recently demonstrated [69]. The role of N-terminal residues in regulating DAT function was also reported in other studies [70] [71].

Pin1-catalyzed cis/trans isomerization is crucial as it allows the dephosphorylation by conformation-specific phosphatases such as PP2A (Fig.7). This was demonstrated in the hyperphosphorylated tau protein. Pin1 facilitates the dephosphorylation of hyperphosphorylated tau protein by PP2A showcasing the presence of a post-phosphorylational regulatory step that restores the function [72] [73]. This indicates an additional regulatory step for proline-directed phosphorylation sites that might dictate DAT function or subcellular localization.

Purpose of the current study

DAT phosphorylation and function has been shown to be regulated by psychostimulant drugs [64,74], [76–78]. Previous studies from our lab indicated a differential regulation by psychostimulant drugs on DAT function and phosphorylation [74], [77]. While direct regulation of DAT by PKC has been reported [45], [78] , MAPK regulation of DAT via dopamine 2 receptors (D_2Rs) was reported [56], [57]. In this study we specifically investigated the effect of psychostimulant drugs and DA on pT53 in a heterologous expression system, rat striatal synaptosomes and *in vivo*. These results will

help in understanding the role of psychostimulant action on pT53 and direct regulation of DAT by MAPK. Also, we designed our experiments to test for post-phosphorylational regulation at pT53 and its effect on DAT function using the Pin1 inhibitor, juglone (Jug). These results will increase our understanding of the additional pathways regulating DAT phosphorylation and function.

CHAPTER II

MATERIALS AND EXPERIMENTAL METHODS

Materials

Animals

Male Sprague Dawley rats (175-300g) were purchased from Charles Rivers Laboratories (Wilmington, MA) and maintained in compliance with the guidelines established by the University of North Dakota Institutional Animal Care and Use Committee and the National Institutes of Health.

Reagents

Protein A Sepharose beads, and High Range Rainbow Molecular Weight Markers were purchased from GE Healthcare Life Sciences (Piscataway, NJ). (-)-Cocaine, d-amphetamine, (+)-methamphetamine, dopamine, and benztropine were purchased from Sigma Aldrich (St. Louis, MO). Phorbol-12 myristate-13 acetate (PMA) and Okadaic Acid (OA), and Juglone were purchased from Calbiochem/EMD Biosciences (La Jolla, CA). Ez-Link Sulfo-NHS-SS-Biotin and immobilized Neutravidin Beads were obtained from Pierce Chemical (Rockford, IL). Complete Mini Protease Inhibitor was purchased from Roche Applied Science (Indianapolis, IN). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO) or Fischer Scientific (Pittsburg, PA).

Equipment

Centrifuges

The Beckman Avanti J-25 was used for synaptosomal preparation. The Beckman J6-MI swinging bucket rotor was used for crosslinking Protein A Sepharose beads. A refrigerated Beckman Microfuge R or a bench top Microfuge were used to pellet cells and for immunoprecipitations.

Electrophoresis

SDS-PAGE was performed using Bio-Rad Mini-Protean III electrophoresis apparatus and proteins transfers to PVDF were performed using Bio-Rad Mini trans blot electrophoresis transfer cell. A Gibco/BRL Life Technologies 250 EX power supply was used to control both Bio-Rad electrophoresis and Bio-Rad protein transfer apparatus.

Cell culture and Miscellaneous

LLC-PK_1 (Lewis lung carcinoma porcine kidney) cells stably expressing rDAT were maintained in a Nuair 2700-30 water-jacketed CO_2 incubator and handled in a Nuair Class II type A/B3 laminar flow hood. Synaptosomal preparations for uptake and efflux assays were handled and washed using the Brandel Tissue Harvester.

Experimental methods

T53 Phosphorylation assay in rDAT LLC-PK_1 cells

LLC-PK_1 cells stably expressing WT rDAT were maintained in α-minimum essential medium supplemented with 10% fetal bovine serum, 2 mM L- glutamine, 200 µg/ml G418, and 1 X penicillin/streptomycin in an incubation chamber with 5% CO_2, 95% O_2 at 37°C. Cells were plated in either 6 or 12 well plates and grown to 80% confluence. Cells were washed twice with 2 ml or 1 ml of Kreb's-Ringer HEPES (KRH) buffer (25 mM HEPES, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM glucose, pH 7.4). Cells were treated with 10 µM d- amphetamine, BZT, 100 µM cocaine, 1 µM OA, 1 µM PMA or vehicle for 30 min at 37° C. BZT, d-amphetamine, (-)-cocaine, were prepared in distilled/deionized water, OA and PMA were prepared in DMSO with a final DMSO concentration maintained below 1%. Each condition was performed in duplicate. Cells were immediately placed on ice and washed with ice-cold KRH twice to remove the drugs. 300 µl of RIPA with protease inhibitor was added to each well and solubilized on ice for 20 min with shaking. The cell lysates were centrifuged at 15,000 x g for 30 min at 4°C. The supernatants were collected and subjected to SDS-PAGE and the proteins were transferred to PVDF membrane. The membrane was immunoblotted with a phospho-specific antibody (1:1000 dilution in 3% BSA blocking buffer) developed against T53 on DAT (pT53 Ab) [69]. Total DAT in the corresponding samples was analyzed by immunoblotting samples with DAT specific monoclonal antibody (1:1000 dilution in 3% BSA blocking buffer) developed against

epitope 16 on the N-terminus on DAT (MAb16) [79]. Parental cells not expressing DAT were used as negative control.

Striatal synaptosomal preparation

Striatal synaptosomes were prepared using male Sprague Dawley rats (175–300 g) [80]. In brief, animals were decapitated and the striatum was removed and weighed. The striata were suspended in cold sucrose phosphate (SP) buffer (10 mM Na_2HPO_4, 0.32 M sucrose, pH 7.4) and homogenized in a Teflon-glass homogenizer. The homogenate was centrifuged at 3000 × g for 3 min at 4°C. The resulting supernatant was further centrifuged at 17000 × g for 12 min at 4°C. The synaptosomal pellet was then re-suspended in 0.32 SP buffer at 20 - 50 mg/ml of original wet weight (OWW) for phosphorylation, uptake and efflux assays.

Phosphorylation, Immunoprecipitation and Immunoblot analysis in striatal synaptosomes

The synaptosomes were aliquoted into individual tubes and treated with vehicle or 10 μM d-amphetamine, (+)-methamphetamine, dopamine, 5μM juglone or indicated concentration for 30 min or the indicated time at 30°C. Each condition was performed in duplicate. The reaction was quenched by adding 1X sample buffer. The samples (50 μl) were then immunoprecipitated with 50% slurry of pT53 Ab crosslinked protein A sepharose beads at 4°C overnight and the beads were washed with IP buffer (phosphate buffered saline, pH 7.4, plus 0.05% SDS, 0.1% Triton X-100) 4 times. The samples eluted with 1X sample buffer at 37°C were subjected to SDS-PAGE electrophoresis and transferred to PVDF. The immunoblot was then developed with MAb16 and pT53Ab for total DAT levels and T53 phosphorylation, which were analyzed by densitometry.

Dopamine uptake in striatal synaptosomes

For uptake assays, synaptosomes were treated with the indicated concentration of Jug or vehicle prior to the assay for 30 min or indicated times at 30°C. The DMSO concentration was maintained ≤ 1%. Transport was initiated by adding the synaptosomes into tubes containing modified-Krebs phosphate buffer (126 mM NaCl, 4.8 mM KCl, 16 mM potassium phosphate, 1.4 mM $MgSO_4$, 10 mM glucose, 1.1 mM ascorbic acid, and 1.3 mM $CaCl_2$; pH 7.4), and [^{3}H]DA to a final concentration of 1 nM. Uptake was carried out in quadruplicate for 3 min at 30°C using 100 μM (−)-cocaine to define non-specific uptake. Transport was stopped by the addition of 5 ml ice-cold SP buffer and synaptosomes were harvested using a Brandel tissue harvester and Whatman GF/B filters pre-soaked for 1 h in a 0.05% polyethyleneimine. Bound radioactivity was quantified by liquid scintillation counting.

Efflux measurement in striatal synaptosomes

Synaptosomes were loaded with [^{3}H]DA and incubated at 30°C for 5min. The extra-synaptosomal [^{3}H]DA was removed by centrifugation of the synaptosomes at 17000 x g for 12 min at 4°C and the supernatant was discarded. The synaptosomal pellet was reconstituted with SP buffer and treated with indicated concentration of juglone for the indicated times at 30°C. The synaptosomes were again spun down and the supernatant was counted for radioactivity. Synaptosomes treated with 100 μM (−)-cocaine along with juglone were considered non-specific efflux.

In vivo analysis treatment of male Sprague-Dawley rats

Male Sprague-Dawley rats were subcutaneously injected with saline or METH (15 mg/kg) or COC (15 mg/kg) and the treated animals were caged separately. The animals were decapitated after the treatment times and brain dissection was performed to remove the striata which were placed in ice-cold SP buffer.

Striatal membrane preparation

Striatal membranes were prepared as described previously [59]. Briefly the membranes were prepared using polytron and the homogenate was then centrifuged at 12000 x g for 12 min at 4°C. The membranes were then immunoprecipitated with protein-A sepharose cross-linked with pT53 Ab overnight at 4°C to avoid any non-specific immunostaining. The eluted sample is then subjected to SDS-PAGE followed by transfer to PVDF membrane. The membrane is then immunoblotted with MAb16 to analyze for T53 phosphorylation (pT53).

ELISA

For the bait, recombinantly expressed and purified NDAT (10µg/ml) and commercially available recombinant Pin1 (10µg/ml) from were coated on ELISA plates overnight at 4°C. The wells are then blocked with commercially available blocking solution at 4°C. The solution containing prey either NDAT (10µg/ml) or Pin1 (10µg/ml) were incubated in the appropriate well. The wells were incubated with MAb16 for NDAT or Pin1 polyclonal Ab and the interaction is detected by incubating with 1mg/ml PNPP substrate. The absorbance was detected at 470 nm.

Statistical analysis

The immunoblots were quantified by densitometry (LumiAnalyst software) and the averaged values were statistically analyzed by student's t-test or ANOVA. The graphs were made in PRISM 3.0 software (Graphpad Software, San Diego CA.).

CHAPTER III

RESULTS

Physiological regulation of DAT T53 phosphorylation

To investigate the physiological regulation of pT53, LLC-PK_1 cells stably expressing rDAT were treated with the protein phosphatase inhibitor, okadaic acid (OA) and PKC activator, PMA. Immunostaining with pT53 Ab revealed that T53 undergoes basal phosphorylation and both OA (178 ± 15%, $p<0.005$) and PMA (140 ± 13%, $p<0.05$) treatments stimulate pT53 (Fig.8). This indicates the dynamic regulation of T53 under physiological conditions. Immunostaining with MAb16 displayed similar DAT levels in the corresponding samples indicating that the treatment had no impact on total DAT protein. The parental LLC-PK_1 cells displayed no immunostaining with both pT53Ab and MAb16 indicating the specificity of the antibodies to DAT [69].

OA dose response in rDAT LLC-PK_1 cells

To identify the protein phosphatase that dephosphorylates pT53 on DAT, we performed an OA dose response treatment in rDAT LLC-PK_1 cells and the cell lysates were analyzed with pT53 Ab or MAb16. An OA dose range of 0.1 nM to 1000 nM was used to treat the cells. The pT53 level increased in a dose-dependent manner with small

Figure 8. Kinase and phosphatase modulators stimulate pT53

Cells expressing rat DAT were treated with 1 μM OA, 1 μM PMA or vehicle (Basal) for 30 min at 37°C. Blots were probed with probed with pT53Ab (top) or MAb16 Ab (bottom). The histogram indicates the quantification of pT53 signal induced by OA and PMA (means ± S.E.), n=3, ***p value <0.0005, t-test. Solubilized DATs were resolved by SDS-PAGE and transferred to PVDF. Blots were probed with pT53Ab or MAb16.

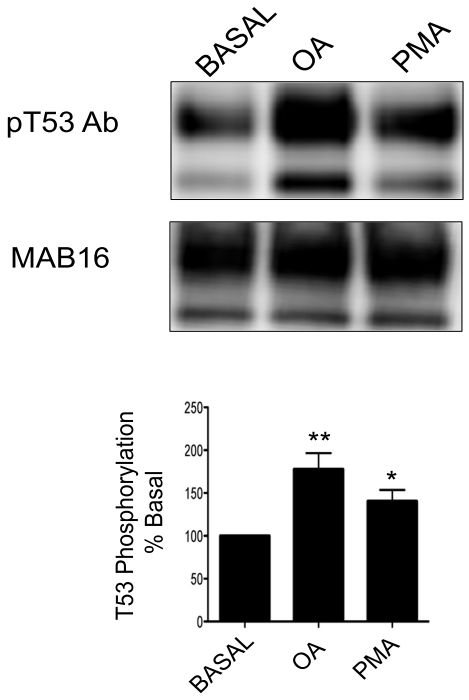

Figure 8. Kinase and phosphatase modulators stimulate pT53

Figure 9. Dose response of OA-stimulated DAT pT53

rDAT LLC-PK_1 cells were treated with increasing doses of OA for 30 min at 30°C. Solubilized DATs were resolved by SDS-PAGE and transferred to PVDF. Blots were probed with pT53Ab (top) to detect phosphorylation and MAb16 (bottom) to detect total DAT. The graph indicates T53 phosphorylation normalized to total DAT with respect to the dose of OA. Arrows indicate the IC_{50} values of the specified phosphatases. n=3, $p < 0.01$ (**) relative to controls by student's t-test.

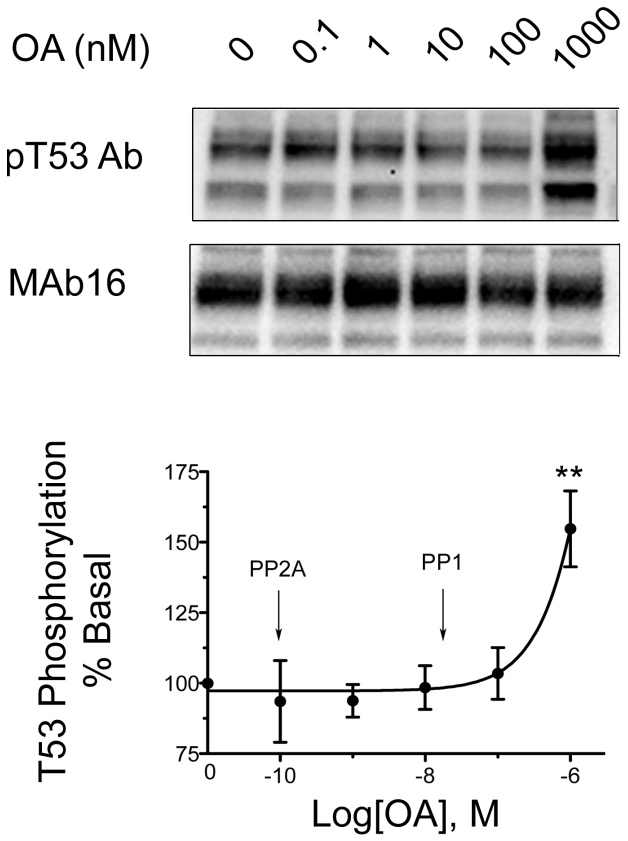

Figure 9. Dose response of OA-stimulated DAT pT53

or no effect at lower doses of OA. According to the dose response curve the IC_{50} of OA for T53 on DAT was indicated to be in the range of phosphatase PP1 (0.15 nM) (Fig.9). Under our experimental conditions no visible effect was observed at IC_{50} values corresponding to other phosphatases indicating PP1 as the potential phosphatase dephosphorylating T53.

Psychostimulants differentially affect T53 phosphorylation on DAT

Psychostimulant drugs have been demonstrated to affect DAT function by regulating DAT phosphorylation [77] [59]. To examine the effects of psychostimulant DAT blockers and substrates on DAT pT53 levels, LLC-PK_1 cells stably expressing rDAT were treated with indicated drugs for 30 min using the phosphatase inhibitor, OA as positive control (Fig.10). Cell lysates were immunoblotted with pT53 Ab, a phospho-specific antibody to detect phosphorylation of DAT specifically at T53 [69]. Western blot analysis revealed that DAT displays pT53 under basal conditions as indicated in lane 1. We observed that the psychostimulant substrate AMPH (lane 4) significantly stimulated the DAT pT53 152 ± 9% of basal, ($p<0.05$) (Fig.10). Inhibition of protein phosphatases with OA resulted in significant accumulation of pT53 levels, 177 ± 19% of basal, ($p<0.005$) (Fig. 8 and 9). Interestingly, DAT blockers COC (110 ± 15% of basal) (lane2) and BZT (93.5 ± 12.5% of basal) (lane 3) had no effect on pT53. The inability of both psychostimulant (COC) and non-psychostimulant (BZT) DAT blockers to stimulate pT53 suggests that the stimulation of pT53 might be a substrate-specific effect. These results demonstrate a differential response of psychostimulant substrates and blockers on DAT pT53. Total DAT levels in the corresponding samples as recognized by MAb16 were

Figure 10. Amphetamine stimulates phosphorylation at T53 on DAT

rDAT LLC-PK_1 cells were treated with vehicle or 10 μM AMPH, BZT or 100 μM COC or 1 μM OA for 30 min and cell lysates were immunoblotted with anti-pT53 Ab to detect phosphorylation or MAb 16 to detect total DAT. The histogram shows pT53 staining normalized for total DAT (means ± S.E.). $n \geq 3$, **p value< 0.0001 relative to basal.

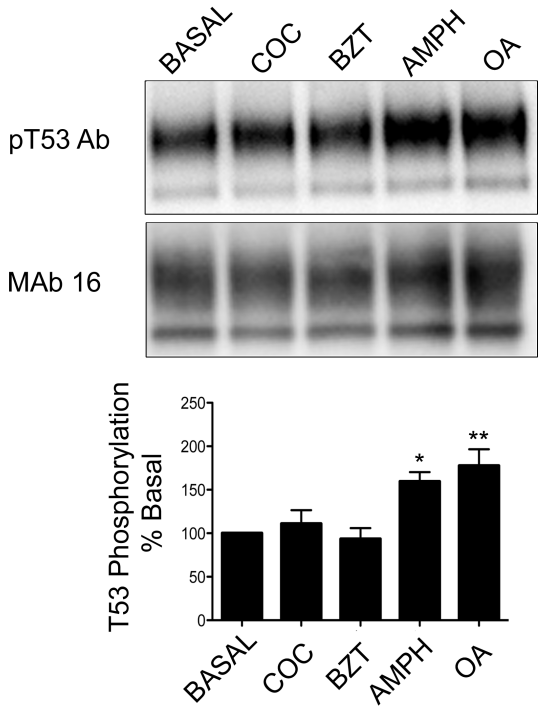

Figure 10. Amphetamine stimulates phosphorylation at T53 on DAT

observed to be unaffected by the treatment conditions.

Psychostimulant substrates stimulate DAT T53 phosphorylation in a cocaine-dependent manner

As DAT uptake blockers displayed no stimulatory effect on pT53 (Fig.10), we questioned if stimulation of pT53 is a substrate-specific phenomenon. To investigate this, we treated rDAT LLC-PK_1 cells with the psychostimulant substrates AMPH, METH and the endogenous substrate, DA for 30 min. The psychostimulant substrates stimulated pT53 significantly, METH (159 ± 24% of basal, $p<0.005$); AMPH (142.4 ± 8% of basal, $p<0.05$) as shown in Fig. 11. The endogenous substrate, DA showed a trend towards increase in pT53 levels (125 ± 6% of basal) however, it was not found to be statistically significant. These data indicated that the increase in DAT pT53 is a substrate-specific phenomenon, with a significantly stronger effect observed with psychostimulant substrates.

Amphetamines are lipophilic in nature and in addition to being transported via DAT into cell, they can also diffuse through plasma membrane [81]. The next question we addressed was, if the effect of AMPH on pT53 is a DAT-mediated effect. We addressed this question by pretreating rDAT LLC-PK_1 cells with 100 μM (-) cocaine, followed by treatment with 10 μM AMPH therefore, preventing the entry of AMPH via DAT. We observed the stimulatory effect of AMPH on pT53 (142 ± 8%, $p<0.005$) (Fig.10 & 11). Similar to the observation in Fig.10, COC treatment alone on rDAT LLC-PK_1 cells did not cause any significant change in pT53 level (110 ± 15%) (Fig.12). In

Figure 11. Psychostimulant substrates stimulate pT53

rDAT LLC-PK_1 cells are treated with vehicle or 10 μM of the indicated drugs for 30 min at 37°C. The cell lysates were immunoblotted with anti-pT53 (top) to detect phosphorylation or MAb 16 (bottom) to detect total DAT. Histogram indicates the T53 phosphorylation normalized to total DAT (means ± S.E.). n=4, $p<0.01$ (**), <0.05 (*), One-way ANOVA, with Tukeys' post-hoc test.

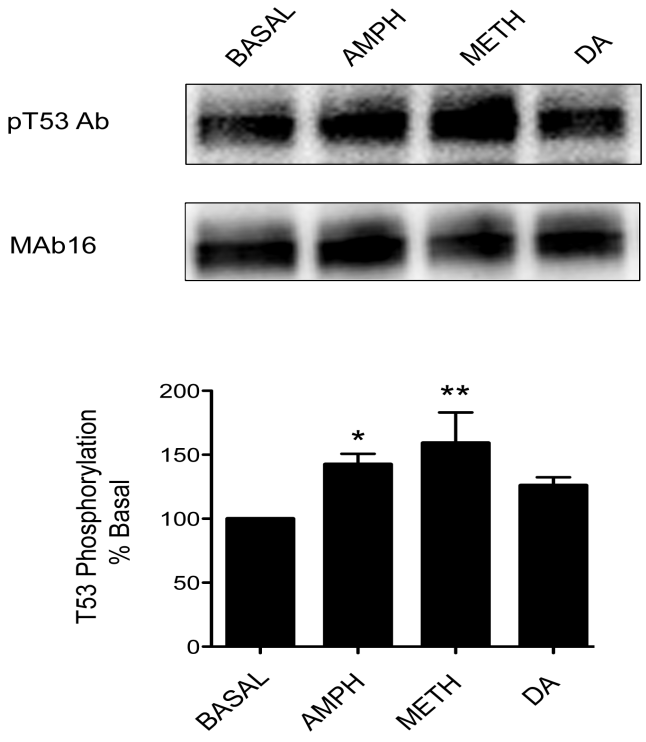

Figure 11. Psychostimulant substrates stimulate pT53

Figure 12. Cocaine blocks amphetamine stimulated DAT pT53

rDAT LLC-PK_1 cells were treated with vehicle or 10 μM AMPH for 30 min in the absence or presence of 100 μM COC. COC pre-treatment was performed for 10 min followed by additional 30 min with AMPH, and cell lysates were immunoblotted with anti-pT53 to detect phosphorylation or MAb16 to detect total DAT. The histogram shows the pT53 staining normalized for total DAT (means ± S.E.). n≥3, ***p value <0.001relative to basal, One-way ANOVA, with Tukeys' post-hoc test.

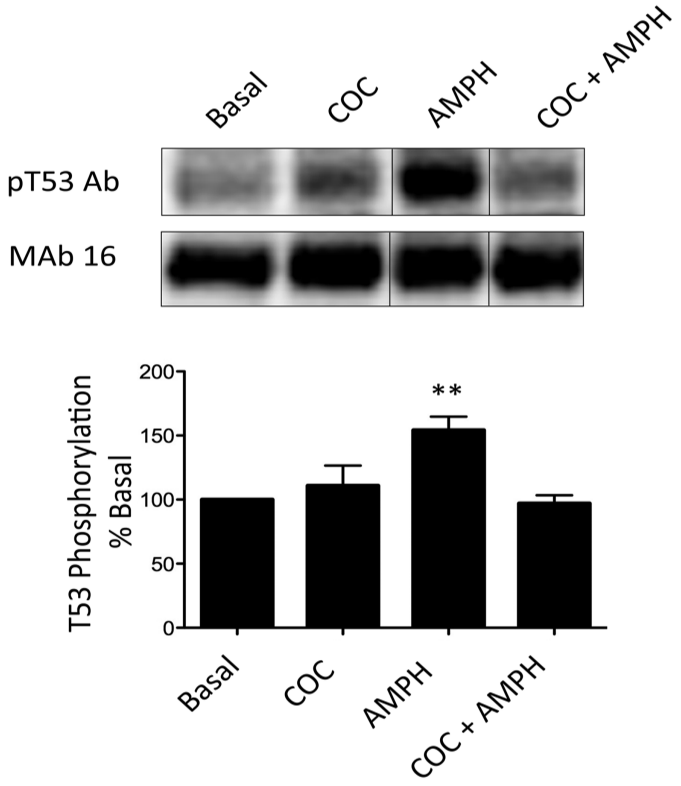

Figure 12. Cocaine blocks amphetamine stimulated DAT pT53

four independent experiments, each performed in duplicate, we observed that the AMPH effect on pT53 was blocked when the cells were pre-treated with COC, displaying 97 ± 6 % of the basal pT53 level. Perturbing the entry of AMPH into the cell through DAT, but not the diffusion through plasma membrane completely abolished the AMPH effect on pT53. This showed that stimulation of pT53 by AMPH is a DAT-mediated effect.

Native tissue response differs from heterologous system in amphetamine effect

Psychostimulant substrates stimulate pT53 in a time-dependent fashion in cells

To analyze the time dependence of AMPH and METH on DAT pT53, we treated LLC-PK_1 rDAT cells with 10 μM AMPH or METH for the indicated time points and the cell lysates were assayed for pT53. AMPH significantly increased pT53 at 30 min (142±7.8% of basal, $p<0.005$) treatment and pT53 level was sustained for atleast 60 min (139±7.6% of basal, $p<0.05$) (Fig.13A). Shorter time points tested (2, 5, 10, min) did not show a significant effect (not shown). METH treatment stimulated a small yet significant increase in pT53 at 2 min (121±7.1% of basal, $p<0.05$), and 30 min METH treatment stimulated pT53 (131±4% of basal, $p<0.005$) (Fig.13B) to levels similar to that of AMPH in cells.

Time course of METH-stimulated pT53 in rat striatal synaptosomes

Next, we sought to investigate the time dependence of stimulated pT53 levels in native tissue. We performed a time course of METH treatment under *ex vivo* conditions, in rat striatal synaptosomes. In contrast to, LLC-PK_1 cells, METH treatment in rat striatal synaptosomes produced a significant increase in pT53 within 60 sec (132 ± 3% of basal,

Figure 13. Time course of amphetamine stimulated pT53

rDAT LLC-PK_1 cells treated with vehicle or 10μM AMPH (A) or METH (B) for the indicated times, were immunoblotted with pT53 Ab to detect phosphorylation or MAb 16 to detect total DAT. The graphs indicate summary of pT53 normalized to total DAT (means ± S.E.). n≥3, **p value <0.005 relative to basal, *p value < 0.05 relative to basal, one-way ANOVA, with Tukeys' post-hoc test.

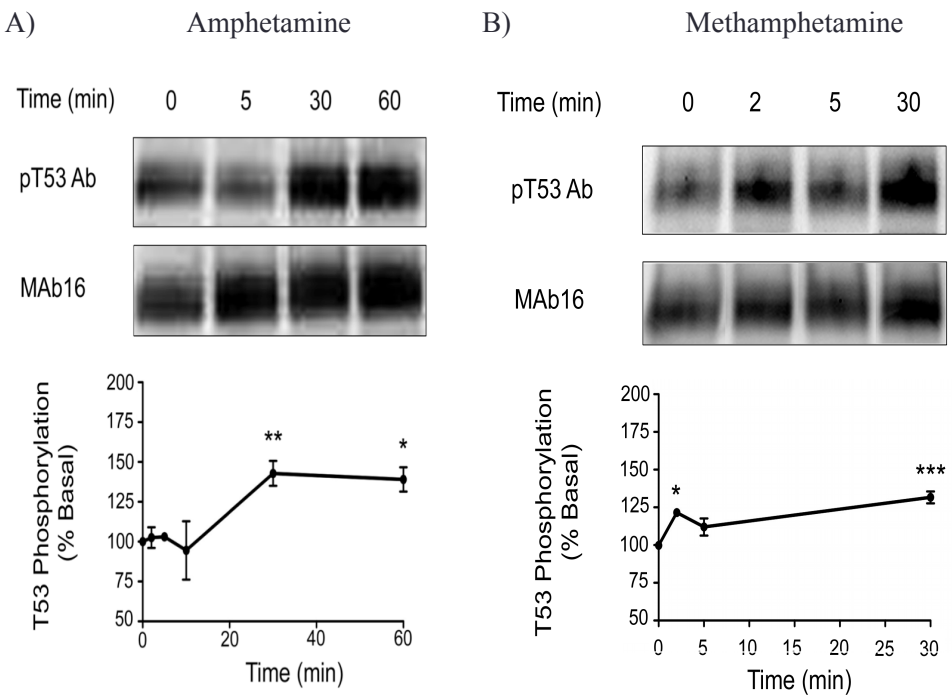

Figure 13. Time course of amphetamine stimulated pT53

Figure 14. Methamphetamine stimulates pT53 in rat striatal synaptosomes

Rat striatal synaptosomes were isolated and treated with 10 μM METH for the indicated times at 30°C. Solubilized DATs were resolved by SDS-PAGE and transferred to PVDF. Blots were probed with pT53Ab. Graph indicates T53 phosphorylation normalized to total DAT (means ± S.E.). $n \geq 3$, **p value<0.005, *p value<0.05 relative to basal, one-way ANOVA, with Tukey's post-hoc test.

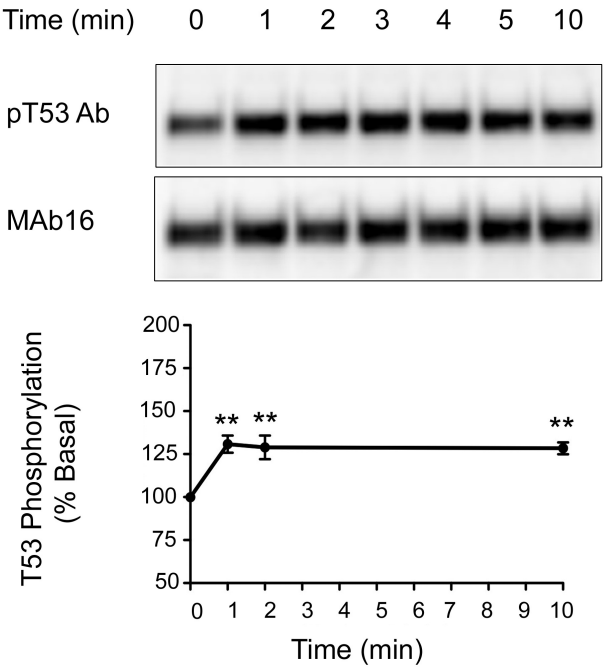

Figure 14. Methamphetamine stimulates pT53 in rat striatal synaptosomes

$p<0.005$) after treatment and this increase was also observed at 10 min (128 ± 3% of basal, $p<0.005$) (Fig.14), and the pT53 level sustained atleast 20 min (121 ± 7%) (data not shown). This indicates psychostimulant drugs impart their effect on pT53 faster in native tissue when compared with the heterologous expression system.

Methamphetamine but not cocaine stimulates pT53 *in vivo*

To investigate the differential regulation of psychostimulant drugs on pT53 *in vivo,* we subcutaneously injected male Sprague-Dawley rats with 15 mg/kg METH; or COC or saline for 30 min (Fig.15). After 30 min, the animals were then decapitated to remove the striata and the striatal membranes were analyzed for pT53 levels and total DAT levels. The saline injected animal displayed basal pT53 levels. COC-injected animals displayed no effect on pT53 levels (Fig.15) similar to our results observed in the heterologous expression system (Fig.13). METH-treated animals displayed a significant stimulation of pT53 (Fig.15) compared to saline and COC injected animal. This further confirms the differential regulation of psychostimulant drugs on pT53 both in a heterologous expression (Fig.13) system and *in vivo*. MAb16 immunostaining displayed similar DAT levels in saline and drug-injected animals indicating that treatment had no effect on total DAT levels.

Methamphetamine time course *in vivo*

To further understand the effect of METH on pT53, we performed a time course of METH treatment *in vivo* in male Sprague-Dawley rats. The animals were subcutaneously (SC) injected with METH (15mg/kg) or saline and caged individually until decapitation. After 10, 30 or 60 min post injection, the animals were decapitated and the brain was

Figure 15. Methamphetamine but not cocaine stimulates pT53 *in vivo*

Male Sprague-Dawley rats were subcutaneously injected with 15mg/kg of methamphetamine (METH), 15 mg/kg cocaine (COC) or saline for 30 min. The animals were decapitated and the brains were dissected to make striatal membranes which then were subjected to immunoprecipitation with protein-A-sepharose cross-linked with pT53 Ab and the eluted sample was resolved by SDS-PAGE and transferred to PVDF. Blots were probed with MAb16. Total DAT levels were determined by directly blotting lysates with MAb16. The DAT pT53 was normalized to total DAT levels. Histogram indicates the quantification of the immunoblots, (means ± S.E.). n=3, *p value<0.05 relative to basal, one-way ANOVA, with Tukey's post-hoc test.

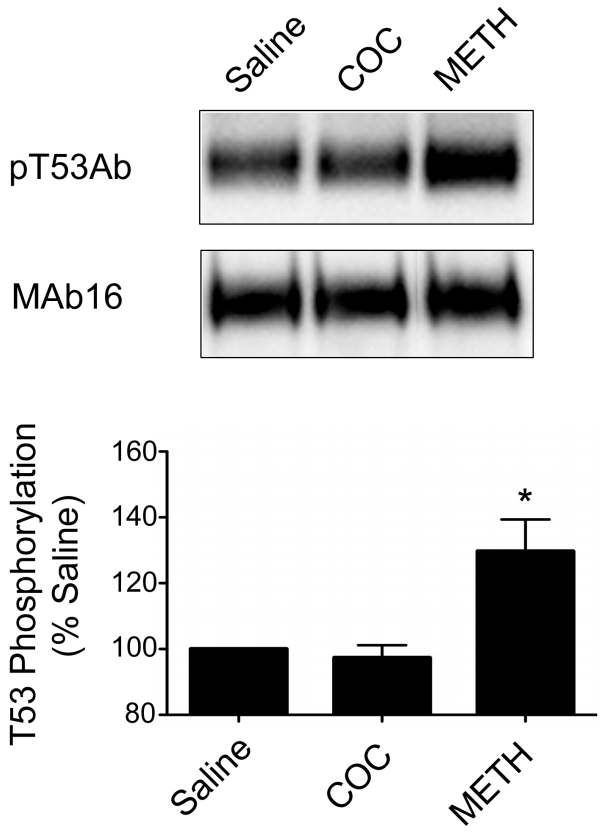

Figure 15. Methamphetamine but not cocaine stimulates pT53 *in vivo*

Figure 16. Methamphetamine stimulates pT53 *in vivo* in a time-dependent manner

Male Sprague-Dawley rats were subcutaneously injected with 15mg/kg of Methamphetamine (METH) for the indicated time points. The animals were decapitated and the brains were dissected to make striatal membranes which then were subjected to immunoprecipitation with protein-A-sepharose cross-linked with pT53 Ab and the eluted sample was resolved by SDS-PAGE and transferred to PVDF. Blots were probed with MAb16. The DAT pT53 was normalized to total DAT levels. Histogram indicates the quantification of the immunoblots, (means ± S.E.). $n \geq 3$, **p value<0.005, *p value<0.05 relative to basal, t-test.

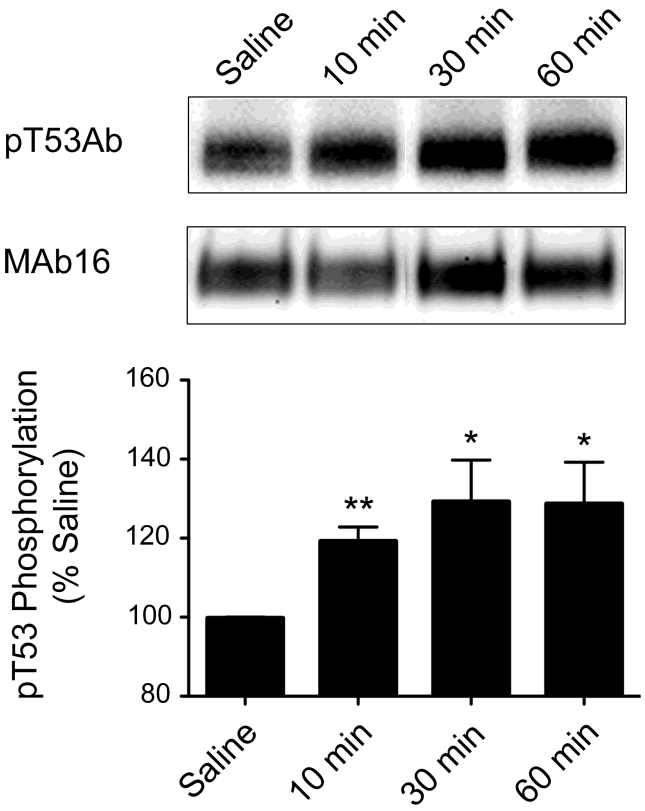

Figure 16. Methamphetamine stimulates pT53 *in vivo* in a time-dependent manner

dissected to remove the rat striatum and the rat striatal membranes were analyzed for pT53 levels. The saline control animals displayed a basal level of pT53 immunostaining similar to the rDAT LLC-PK_1 cells indicating the regulation of pT53 under physiological conditions. We observed the stimulation of pT53 levels in METH-injected animals and this effect occurred in a time-dependent fashion. The shortest *in vivo* time point tested was 10 min, which displayed a significant stimulation of pT53 at 10 min (119 ± 3%, $p<0.005$). The stimulation of pT53 was further increased at 30 min (129 ± 10%, $p<0.005$) and the increased pT53 was sustained at 60 min (128 ± 10%, $p<0.05$) (Fig.16) indicating the alteration of downstream signaling. The METH-injected animals were observed to be visibly hyperactive compared to the saline control.

Pin1 inhibitor, juglone, stimulates pT53 in cells and in rat striatal synaptosomes

We hypothesized that Pin1 isomerizes cis-pThr53-Pro54 to trans-pThr53-Pro54 allowing dephosphorylation by conformation-specific phosphatases. To investigate our hypothesis of Pin1 regulation of DAT, we used juglone (Jug), a small-molecule inhibitor specific for Pin1. LLC-PK_1 cells stably expressing rDAT were treated with Jug or DMSO as control and the cell lysates were analyzed for pT53 and total DAT levels. Treatment of LLC-PK_1 cells stably expressing rDAT with Jug significantly increased pT53 at both 5 μM (162 ± 13%, $p< 0.005$) and 10 μM (128 ± 12%, $p<0.05$) (Fig.17) compared to the control conditions. OA used as positive control stimulated pT53 120 ± 5%, $p<0.005$ compared to control. Jug-stimulated pT53 represents the accumulation of the cis-conformation of the pThr53-Pro54 sequence that is inaccessible to the trans-conformation

Figure 17. Pin1 regulates dephosphorylation of DAT T53 in LLC-PK_1 cells and in rat striatal synaptosomes

(A) rDAT LLC-PK_1 cells or (B) rat striatal synaptosomes were treated with indicated concentrations of Pin1 inhibitor, Juglone for 30 min at 30°C. The samples were then solubilized with sample buffer and DAT was resolved with SDS-PAGE and transferred to PVDF membrane. The pT53 signals were identified with pT53 Ab and total DAT levels with MAb16. Histogram indicates phospho-T53 signals normalized to total DAT (means ± S.E.), n=3, ** $p<0.005$, ANOVA with Tukey's post hoc test).

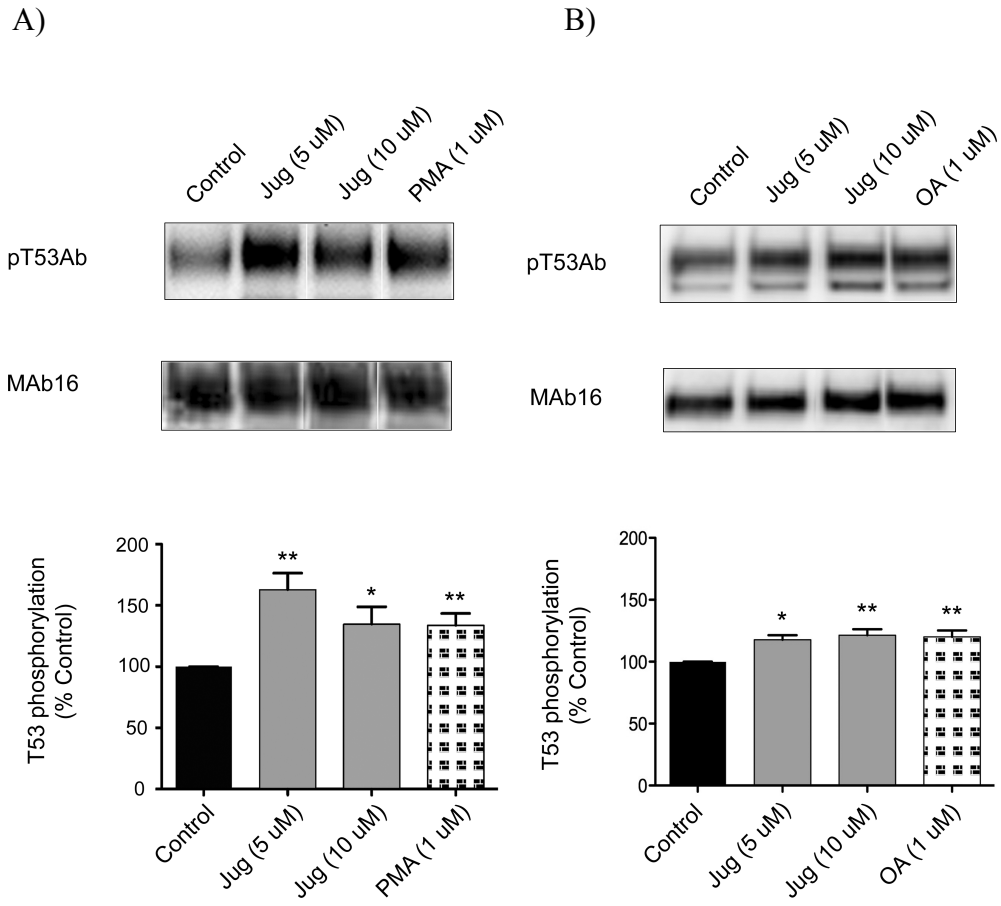

Figure 17. Pin1 regulates dephosphorylation of DAT T53 in LLC-PK$_1$ rDAT cells and in rat striatal synaptosomes

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preferring phosphatases. This indicates post-phosphorylation regulation of DAT by the prolyl isomerase, Pin1.

We investigated if Pin1 exhibited similar regulation on DAT in native tissue. To analyze that, we treated rat striatal synaptosomes with Juglone or DMSO and measured pT53 levels. The DMSO control revealed basal-level pT53, which was significantly increased in the presence of Jug, both at 5 μM, 117 ± 3 %, $p<0.05$ and at 10 μM, 121 ± 4 %, $p<0.005$ compared to control. Activation of protein kinase C (PKC) by PMA, used a positive control also stimulated pT53 133 ± 9 %, $p<0.005$, compared to control (Fig.17). The total DAT levels were unaffected by the treatment conditions. This indicates the Pin1 regulates DAT pT53 in heterologous expression system and in rat striatal synaptosomes. Our data reveals a novel pathway of DAT regulation by Pin1 via T53 phosphorylation.

Juglone stimulates [^{3}H]DA efflux from rat striatal synaptosomes

We next explored the effect of Pin1 regulation on DAT function. Rat striatal synaptosomes were treated with Jug and analyzed for basal [^{3}H]DA efflux. We observed a basal [^{3}H]DA efflux in synaptosomes treated with DMSO. Jug treated rat striatal synaptosomes exhibited a significant increase in [^{3}H]DA efflux both at 5 min (211 ± 25%, $p<0.005$) and 15 min (203 ± 23%, $p<0.005$) (Fig.18) treatment time points compared to the control conditions. The efflux observed was blocked by COC and occurred in the absence of efflux-stimulating substances like amphetamine (AMPH). This is the first observation to demonstrate a significant increase in basal efflux by a cis-trans prolyl isomerase inhibitor. We observed that under the same conditions, DAT uptake activity from rat striatal synaptosomes was not significantly altered by Jug (data not

shown) suggesting that Jug effect on DAT function could potentially be altering DAT conformation to an efflux promoting state.

Juglone stimulates pERK

We next explored the pathway of Jug-stimulated efflux in rat striatal synaptosomes. Jug stimulates pT53 (Fig.17) in rat striatal synaptosomes and T53 has been demonstrated to be a substrate for proline-directed kinases such as ERK [44]. We analyzed the rat striatal synaptosomal sample for increase in phosphorylated ERK (pERK). The rat striatal synaptosomes revealed a basal pERK signal, which was significantly increased in rat striatal synaptosomes treated with Jug for both 5 min (196%±22, $p<0.005$) and 15 min (175±23%, $p<0.05$) compared to DMSO treated control (Fig.19). This suggests that Pin1 also regulates the function of ERK by cis-trans isomerization of proline-directed phosphorylation sites on ERK required for its activation. Immunostaining with ERKAb revealed that total ERK levels were unaffected under the experimental conditions indicating that Jug stimulates pERK without affecting the total ERK levels. This suggests that Jug-stimulated [^{3}H]DA efflux could be achieved via pERK mediated phosphorylation of T53 on DAT.

Pin1 interacts with the N-terminus of DAT

Our next step was to investigate the interaction between Pin1 and DAT. We recombinantly expressed the N-terminus of DAT (N-DAT) in E.coli and purified the peptides as described in [44]. ELISA was performed using N-DAT as bait and recombinant Pin1 as prey and vice-versa. The colorimetric density was used as a measure of interaction between N-DAT and Pin1 (Fig.20). We observed a strong

interaction between N-DAT and Pin1, occurs in a dose-dependent manner (data not shown). Our ELISA data demonstrates the prolyl isomerase, Pin1 as a new interaction partner of DAT (Fig.20), which regulates the mechanism of DAT mediated [^{3}H]DA efflux and also the conformational state of pT53, thereby dictating its dephosphorylation.

Figure 18. Juglone stimulates [^{3}H]DA efflux in rat striatal synaptosomes

Rat striatal synaptosomes were loaded with [^{3}H]DA for 5 min at 30°C followed by treatment with Jug for 5 and 15 min at 30°C. The synaptosomes were immediately centrifuged at 4°C at 17000xg for 12 min and the supernatant was analyzed for [^{3}H]DA efflux. Jug significantly increased [^{3}H]DA efflux from rat striatal synaptosomes at both 5 and 15 min, ** $p<0.005$, n (4), one-way ANOVA, tukey's post-hoc test.

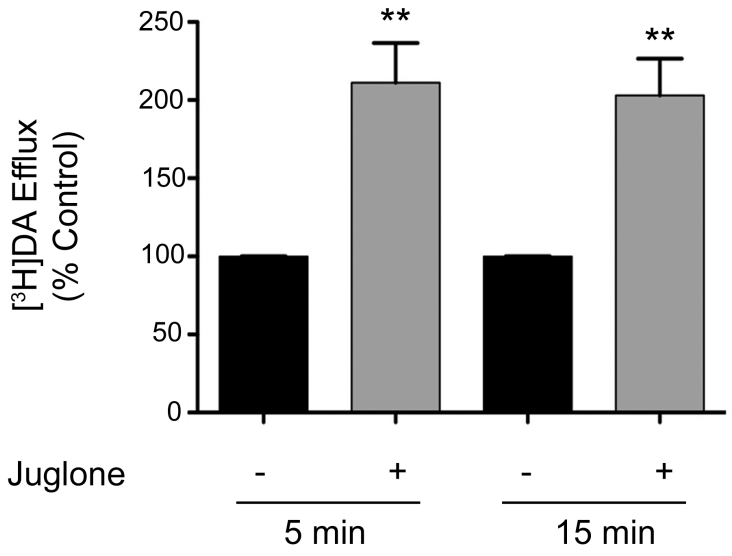

Figure 18. Juglone stimulates [^{3}H]DA efflux in rat striatal synaptosomes

Figure 19. Juglone activates ERK

Jug-treated rat striatal synaptosomes were immunoblotted with pERK Ab and ERK Ab to analyze the levels of activated ERK and total ERK respectively. Inhibition of Pin1 by Jug significantly increased pERK compared to control, ** $p<0.005$ and * $p<0.05$, n (4), one-way ANOVA, tukey's post-hoc test. Histogram demonstrates the quantification of pERK normalized to total ERK.

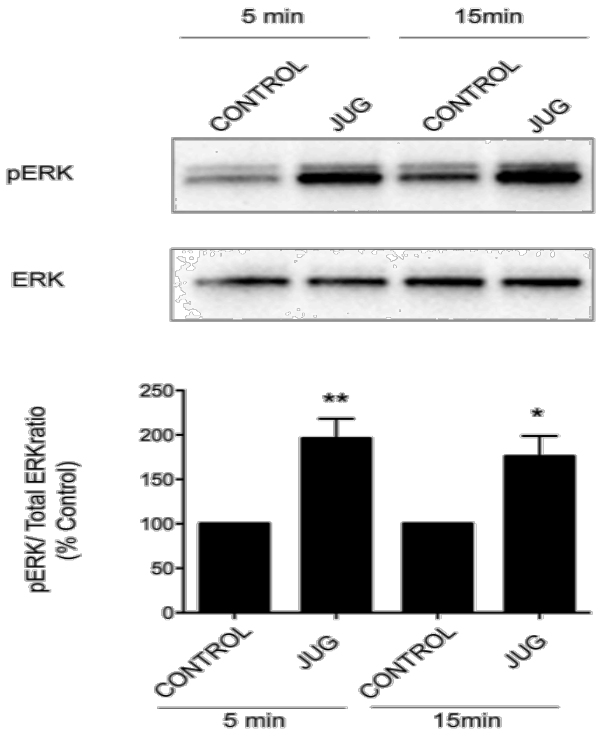

Figure 19. Juglone activates ERK

Figure 20. Pin1 interacts with the N-terminus of DAT

ELISA was performed as described in methods. Briefly, recombinantly expressed N-terminus of DAT (N-DAT, 10 μg/ml) was used as bait and recombinant Pin1, (10 μg/ml) was used as prey. PNPP was used as substrate for colorimetric analysis. The absorbance was measured at 405 nm revealing the interaction between N-DAT and Pin, n=3.

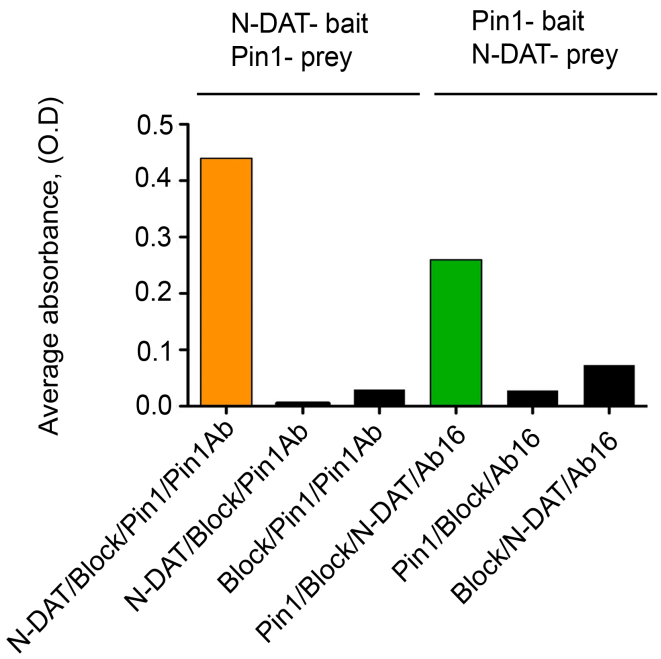

Figure 20. Pin1 interacts with the N-terminus of DAT

CHAPTER IV

DISCUSSION

Psychostimulant substrate regulation of DAT T53 phosphorylation

Our study demonstrates the differential effect of psychostimulant drugs on pT53, a proline-directed phosphorylation site on the DAT N-terminus. Psychostimulant substrates like AMPH and METH were observed to increase pT53 in a heterologous expression system, rat striatal preparations and under *in vivo* conditions. Uptake blockers tested including COC, BZT were not capable of this stimulatory effect nor did they affect basal pT53 levels. Although AMPH and METH have been shown to stimulate PKC-mediated DAT phosphorylation, the majority of which occurs at the serine cluster at the N-terminus [59], this is the first evidence to demonstrate a differential effect of psychostimulant substrates and blockers on the proline-directed phosphorylation site on DAT. Proline-directed kinases such as ERK regulation of DAT activity via dopamine receptor mediated mechanisms [56], [57] have been shown. However, our findings unveil a direct regulation of DAT by proline-directed kinases via T53.

We have also identified a novel regulatory pathway of DAT function by a peptidyl prolyl cis-trans isomerase, Pin1. A small molecule inhibitor of Pin1, Jug significantly increases pT53 in both a heterologous expression system and in rat striatal

synaptosomes. In addition, we also observed that Pin1 regulates the efflux mechanism of DAT in rat striatal synaptosomes potentially via direct interaction between N-DAT and Pin1.

Inhibition of phosphatases by OA has always produced the highest level of DAT phosphorylation indicating the continuous activity of the physiological system to maintain a non-phosphorylated DAT state. In attempts to identify the phosphatase acting at T53 we performed dose response of OA in rat striatal synaptosomes and a heterologous cell system. OA dose response performed in rat striatal synaptosomes and the heterologous system both indicated PP1 to be the likely phosphatase involved in T53 regulation. No effect was seen at lower doses of OA that corresponds to inhibit PP2A ruling out PP2A as the phosphatase acting at T53 under the conditions we tested. PP1 has been shown to be the primary phosphatase involved in DAT phosphorylation regulation although PP2A inhibitor also showed a significant increase in DAT phosphorylation [82]. It is possible that our experimental conditions or detection by western blot was not sensitive enough to detect an effect present at lower doses of OA. Further research with specific phosphatase inhibitors is required to approach this aspect.

The AMPH effect on DAT T53 phosphorylation is either due to binding to or transport through DAT as COC blockade of DAT prior to AMPH treatment in LLC-PK_1-rDAT abolishes the AMPH effect. This is in accordance with the METH effect on the DAT phosphorylation which was also blocked by COC pre-treatment [59]. This indicates that the AMPH effect on DAT phosphorylation is not due to diffusion of AMPH through the plasma membrane but DAT-dependent. COC by itself did not influence both overall DAT phosphorylation (48) as well as T53 phosphorylation.

The time dependence of pT53 stimulated by AMPH in a heterologous expression system and in striatal synaptosomes produced interesting results. AMPH treatment of LLC-PK_1- rDAT showed a significant increase in T53 phosphorylation only after 30 min treatment and was sustained until 60 min. The shorter time points (5-20 min) did not affect the basal T53 phosphorylation. However in rat striatal synaptosomes METH caused a significant increase in DAT T53 phosphorylation as short as 60 sec. This is the first evidence so far for a rapid phosphorylational stimulation by a psychostimulant. METH effect on T53 phosphorylation remained significant until 10 min and the next time point tested (20 min) does not show a significant effect. It is interesting to note that the METH effect on overall DAT phosphorylation which was observed to be on the serine-cluster of the N-terminus is significant only at 10 and 15 min treatment [59]. METH induced DAT T53 phosphorylation might be one of the initial steps followed by serine-cluster phosphorylation. Since the METH-effect on T53 is instantaneous, it might be affecting the transport kinetics or interaction of DAT with other proteins that facilitate or affect function of DAT downstream.

We have previously reported in both the heterologous expression system and in rat striatal tissue that, T53 displays basal level phosphorylation which is stimulated in the presence PMA and OA [69] and by psychostimulant substrates as indicated in our current study (Fig.1, and 6). Our previous studies revealed that T53A rDAT (phosphorylation-deficient mutant) and T53D rDAT (phospho-mimetic mutant), had impaired [^{3}H]DA uptake activity compared to WT rDAT. Also, both T53A rDAT and T53D rDAT displayed no detectable AMPH-stimulated [^{3}H]MPP^+ release [69]. The similar functional pattern of both T53A and T53D rDAT suggested that it is not just the negative charge

imparted by the phosphorylation rather a potential conformational change associated with the pT53 that is crucial for the DAT function. Another N-terminal mutant, T62D DAT has been reported to display altered DA uptake and efflux indicating the structural and functional importance of the N-terminus [70].

Amphetamines are also known to impact the surface expression of DAT in a biphasic manner, which was observed to be cocaine-sensitive. In both rat striatal synaptosomes and a cell system treated with AMPH increased the surface expression of DAT within 30-60 sec and remained till 60 sec along with an increase in efflux [41], [83] but, AMPH treatment for 30 min decreased the surface expression of DAT [40]. METH-induced T53 phosphorylation that fades at later time points might be associated with the biphasic phenomenon observed in the regulation of surface expression of DAT by AMPH.

Neuronal proteins like syntaxin 1A (Syn 1A) have been found to affect DAT function and phosphorylation. Treatment of Syn 1A protease Botulinum Neurotoxin C (BoNT/C) increases DAT phosphorylation in rat striatal tissue along with an increase in uptake [35]. Syn 1A interacts with the N-terminus of DAT (1-33 aa of DAT) which is stimulated in the presence of AMPH [36]. Expression of Syn 1A in hDAT expressing cells potentiated AMPH-stimulated efflux. Interestingly, inhibition of CAMKII, the activity of which was observed to be required for AMPH-stimulated efflux [62], abolished the Syn 1A stimulated AMPH efflux. Such events are also observed in other transporters like NET. The AMPH-stimulated association and surface expression of Syn 1A with NET was inhibited with CAMKII inhibitor [75]. This suggests the AMPH induced phosphorylation might promote the association of proteins like Syn 1A with the

transporters to further regulate transporter function and surface expression. AMPH-induced T53 phosphorylation on DAT might also be following a similar mechanism although further research is required to address if any protein associations are affected.

Recombinantly expressed N-DAT was phosphorylated by PKC, PKA and proline-directed kinases like ERK, JNK and p38. Phosphoamino acid analysis of rDAT indicated that T53 was strongly phosphorylated by proline-directed kinases like ERK, JNK and p38 [44]. Our attempts to identify the proline-directed kinase involved in regulation of T53 phosphorylation in striatal synaptosomes did not provide supporting results. Though the inhibitors used were membrane permeable, we assume that the inhibitors were not accessible in the treatment time or the western blot employed was not sensitive to detect the effects. However it is noteworthy to mention that PKC activator PMA treatment both in heterologous system and rat striatal synaptosomes stimulated the T53 phosphorylation significantly.

Post-phosphorylation control of DAT function by peptidyl prolyl cis-trans isomerase, Pin1

In this study, we identified a novel regulatory pathway of DAT by the peptidyl prolyl cis-trans isomerase Pin1, potentially dictating the cis-trans isomerization of pT53 on the N-terminus of DAT. This is the first study to show DAT regulation by Pin1, which, has been associated with several pathological conditions including cancer and Alzheimer's disease.

Pin1 has been demonstrated to catalyze the isomerization of pSer/Thr-Pro peptides from cis to trans configuration in several proteins. Restoration of

hyperphosphorylated tau (ptau) protein to the trans conformation by Pin1 is required for dephosphorylation, as phosphatases like PP2A have been shown to prefer trans configuration [72]. Consistent with these studies, our data revealed that inhibition of Pin1 using juglone (Jug), thereby preventing cis to trans isomerization, significantly increased the pT53 on DAT in both a heterologous expression system and in rat striatal tissue. The increase in pT53 observed was in the absence of any stimulating conditions like kinase activators or phosphatase inhibitors or psychostimulant substrates. This is the first evidence to demonstrate that isomerization of cis-pT53 to trans-pT53 on DAT by Pin1 is required for dephosphorylation. Recombinantly expressed N-terminus of DAT (N-DAT), phosphorylated by ERK was shown to be resistant to dephosphorylation *in vitro* by either of the phosphatases; PP2A or PP1 [44] consistent with our studies indicating the importance of Pin1 catalyzed post-phosphorylation cis-trans conformational change for dephosphorylation. The major Ser/Thr phosphatase, PP2A has been shown to interact with the N-terminus of DAT [33] in a structural proximity to potentially dephosphorylate T53 on the N-terminus of DAT post the Pin1 catalysis. PP2A whose activity on its substrates is regulated by Pin1 is known to interact with DAT (site), which leads one to speculate a possible effect of Pin1 on DAT.

Proline-directed kinase, ERK has been shown to regulate DAT uptake via DA receptor-mediated mechanism [56], [57]. Treatment of rat striatal synaptosomes with Jug caused a tremendous increase in [^{3}H]DA efflux compared to control conditions. This increased efflux was observed in the absence of amphetamine, a classic inducer of efflux. To our knowledge, this is the first evidence demonstrating a direct role for proline-directed phosphorylation on the DAT efflux mechanism. This finding further confirms

that Pin1 catalyzed conformational change is required for the efflux mechanism as our previous studies have demonstrated the inability of T53D rDAT to efflux [69]. We observed that uptake activity was not altered under the same conditions in rat striatal synaptosomes. Jug stimulated efflux in rat striatal synaptosomes was associated with significant increase in pERK levels compared to control. This suggests that Jug-stimulated efflux observed in the rat striatal synaptosomes potentially could be mediated through pERK phosphorylation of T53 on DAT. We are not ruling out the possibility that Jug-mediated [^{3}H]DA efflux could be through its action on another neuronal protein as Pin1 is known to impact several neuronal proteins like tau, neurofilaments [73], [84], [85] and also other kinases like JNK [86]. In addition, T53 on the N-terminus of DAT is localized in close proximity to TM1, which forms one of the components of the substrate translocation pathway. The pT53 on DAT with its Pin1 catalyzed conformational rearrangement, might impact TM1, the substrate permeation pathway component and the putative intracellular gate residue R60, thereby potentially influencing DAT function.

Our ELISA data indicated interaction of Pin1 with N-DAT peptide (Fig.20). This suggests that the effect of Jug, on [^{3}H]DA efflux mechanism could be due to the direct interaction between Pin1 and N-DAT through isomerization inhibition of pThr53-Pro. The AMPH-stimulated efflux has been shown to be affected by several DAT interaction partners including Syn 1A, and Flotillin [36][39]. Pin1, by regulating the conformation of pThr53-Pro, which also lies in the SH3 domain ligand (P-P-X-X-P), could potentially control the interaction of other scaffolding proteins and their regulation of DAT function. We observed that the total amount of DAT was not affected by Jug under our experimental conditions.

DAT and diseases

Dopaminergic signaling has been implicated in several severe pathological conditions including ADHD (Attention deficit hyperactive disorder), Schizophrenia, Tourette syndrome, bipolar disorder and Parkinson's disease (PD). DAT polymorphisms and death of the dopaminergic neurons are some of the characteristics of a few disease states, strongly implying the importance of dopamine homeostasis for normal physiological function.

In the case of ADHD, a wide set of DAT polymorphisms both in the coding regions and the intronic regions have been reported in humans, some of which are A559V, R615C, V382A, V55A, E602G hDATs [87][88][89]. A bipolar disorder patient and two male siblings diagnosed with ADHD have been reported to have the A559V hDAT mutation [90]. These polymorphisms, when cloned into heterologous expression systems displayed abnormal DAT functionalities. A559V hDAT displayed a ~ 300% increase in spontaneous DA efflux compared to WT DAT. This is referred to as anomalous DA efflux (ADE). This indicates the ability of a point mutation to significantly affect DAT function potentially by altering the conformation of the substrate translocation pathway of DAT.

The role of DA D_2 auto-receptors (D_2R) in mediating the ADE of A559V hDAT was demonstrated in 2010 [87]. The ADE displayed by A559V hDAT is thereby mediated through D_2R activation of CaMKII catalyzed phosphorylation of N-terminal serines [87]. D_2R-mediated DAT regulation by MAPK has been previously demonstrated

[57]. This data demonstrated the involvement of phosphorylation of the DAT N-terminus to play a crucial role in pathological conditions.

R615C DAT, another ADHD associated mutant had significant reduction in surface expression with AMPH-stimulated efflux comparable to WT DAT. Coimmunoprecipitation studies demonstrated that R615C DAT-CaMKII complexes were significantly enhanced relative to WT DAT-CaMKII complexes. The [^{32}P] Labeling of R615C DAT displayed significantly increased basal phosphorylation compared to WT DAT [91].

Pin1 has been shown to promote APP degradation in AD [92] by inhibiting GSK3β thereby reducing the levels of amyloid beta [93]. The notable feature of AD-affected neurons is the depletion of soluble Pin1. Pin1 has been shown to facilitate the dephosphorylation of tau protein by PP2A showcasing the presence of a post-phosphorylation regulatory step that restores the function [72][73]. This indicates an additional regulatory step for phosphoproteins that might dictate its function or subcellular localization.

Our data culminates to the point that Pin1 regulates the DAT efflux mechanism potentially through isomerization of p53 on DAT. All of this experimental evidence extends our understanding of psychostimulant substrate regulation of DAT phosphorylation at the proline-directed phosphorylation site, T53. Also, we revealed a novel pathway of DAT regulation by the isomerase Pin1, which could be exploited to understand DAT regulation under both physiological and pathological states.

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